# Bacterial Responses During Antibiotic Treatment

---

**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde**

**(Dr. sc. nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

**von**

**Paolo Santiago S. Ocampo**

**aus den**

**Philippinen**

Promotionskomitee

Prof. Dr. Leo Eberl (Vorsitz)

Prof. Dr. Sebastian Bonhoeffer (Leitung der Dissertation)

Prof. Dr. Martin Ackermann

Dr. Gabriel Perron

Zürich, 2014

*Para sa aking magulang at mga kapatid*

# Summary

The ability of bacteria to evolve resistance to drugs has been recognized since the discovery of penicillin in the 1920s. Compounded by a slow novel antibiotic development pipeline, the acquisition of resistance by pathogens is so prevalent and widespread that the loss of arguably the most important medical intervention of our period has become imminent. The challenge presented by the epidemic of resistance requires a clinically sufficient strategy that specifically delays the evolution of resistance to safeguard the future utility of our available drugs.

In this dissertation, I approach the problem of resistance from the perspective of bacteria as they are exposed to drugs. I propose that the different responses both sensitive and resistant bacteria are able to produce during antibiotic treatment could help explain how resistance is maintained in the absence of selection, and could aid in the development of treatment strategies that discourage the evolution of resistance.

First, I aimed to describe the relationship between antibiotic concentration and the fitness of specific genotypes. I used a large set of sensitive and resistant bacteria and measured growth and death rates of all the strains in a wide range of drug concentrations. By doing so, I obtained fitness landscapes that were found to be strongly heterogeneous. Combined with a mathematical model, the results produced in this chapter suggest that the strong dependence of bacterial fitness on specific antibiotic concentrations aids in the co-occurrence of both sensitive and resistant strains.

In the following chapter, I describe a novel phenomenon that indicates a connection between resistant bacteria and the antibiotic to which they are resistant. I find that growth of streptomycin resistant bacteria in high concentrations of the drug produces transgenerational fitness benefits in the presence of a

second, unrelated drug. Revealing such effects impacts our understanding of resistance as more than just a mechanism by which bacteria survive treatment, but may also be adaptive in novel environments.

Last, I consider combination therapy as a tool that could be applied to delay the evolution of resistance. As an initial step towards the wider adoption of pairs of drugs in the treatment of acute bacterial infections, I show that a basic pharmacodynamic property of all antibiotics helps predict when an antagonistic interaction can be expected. Manipulating different interactions between drugs has the potential to both increase clinical efficiency as well as control the problem of resistance.

# Zusammenfassung

Schon seit der Entdeckung des Penicillins in den zwanziger Jahren des letzten Jahrhunderts ist bekannt, dass Bakterien Resistenzen gegen Antibiotika entwickeln können. Aufgrund der Häufigkeit und der weiten Verbreitung von Antibiotikaresistenzen und weil die Entwicklung von neuen Antibiotika nur sehr langsam voranschreitet, droht die Gefahr, dass eine der wohl bedeutendsten medizinischen Errungenschaften bald wirkungslos wird. Angesichts dieser Entwicklung braucht es eine klinisch wirksame Strategie, die spezifisch darauf abzielt, die Evolution von Resistenzen zu verlangsamen, um die langfristige Wirksamkeit der vorhandenen Antibiotika zu garantieren.

In dieser Dissertation untersuche ich, wie Bakterien während der Antibiotikabehandlung reagieren. Ich zeige auf, wie die unterschiedlichen Reaktionen von resistenten und sensitiven Bakterien zum einen dabei helfen können, zu verstehen, wie Resistenz auch ohne Selektion erhalten bleibt, und zum anderen zur Entwicklung von Strategien zur Verminderung der Resistenzevolution beitragen könnten.

Zuerst beschreibe ich den Zusammenhang zwischen der Konzentration von Antibiotika und der Fitness von spezifischen Genotypen. Ich habe die Wachstums- und Todesraten einer grossen Anzahl von sensitiven und resistenten Bakterienstämmen innerhalb einer breite Spanne von Antibiotikakonzentrationen gemessen. Die dabei erhaltenen Fitnesslandschaften stellten sich als sehr heterogen heraus. In Kombination mit einem mathematischen Model legen diese Resultate nahe, dass die starke Abhängigkeit der bakteriellen Fitness von der Konzentration des Antibiotikums das gemeinsame Auftreten von sensitiven und resistenten Bakterien ermöglichen kann.

Das nächste Kapitel beschreibt ein neuartiges Phänomen, das eine spezifische Interaktion zwischen resistenten Bakterien und dem Antibiotikum,

gegen das sie resistent sind, andeutet. Streptomycin-resistente Bakterien, die in einer hohen Konzentration des Antibiotikums wachsen, zeigen einen transgenerationellen Fitnesszuwachs, wenn sie in Gegenwart eines zweiten, nicht verwandten Antibiotikums wachsen. Das Aufdecken solcher Zusammenhänge beeinflusst unser Verständnis von Resistenz als einem Mechanismus, der nicht nur das Überleben einer Antibiotikabehandlung ermöglicht, sondern auch eine adaptive Funktion in neuen Umgebungen hat.

Im letzten Kapitel betrachte ich Kombinationstherapie als ein Instrument, das die Evolution von Antibiotikaresistenz verzögern könnte. Als erster Schritt in Richtung eines grossflächigeren Einsatzes von Paaren von Antibiotika für die Behandlung von akuten bakteriellen Infektionen zeige ich, dass eine grundsätzliche pharmakodynamische Eigenschaft aller Antibiotika vorherzusagen hilft, ob eine antagonistische Interaktion zwischen zwei Antibiotika erwartet werden kann. Durch die Manipulation der verschiedenartigen Interaktionen zwischen Antibiotika wird es unter Umständen möglich, sowohl die klinische Effizienz zu erhöhen als auch das Resistenzproblem unter Kontrolle zu bringen.

# Contents

# 1

# Introduction

Antibiotics are arguably the most important medical discovery in human history to date. Since the introduction of antibiotics to the clinic in the 1920s, infections that were commonly fatal are now merely common and prophylactic antibiotic use has made modern surgery possible [1]. Bacteria, however, have kept pace with this innovation and have quickly evolved mechanisms to resist killing by every antibiotic shortly after each drug was made available to patients [2]. Now that the prolific drug discovery period of the 1940s to the 1960s has ended, the problem of antibiotic resistance has caught up and the risk of a pathogen becoming resistant to every drug available has become real [3].

Laboratory studies of resistance have primarily and appropriately focused on the survivors of antibiotic exposure. Identifying the mechanisms of resistance bacteria employ has guided the search for new drugs and informed the design of completely synthetic antimicrobials [4,5]. Currently, every antibiotic on the market targets an essential function in bacteria. This is due to the principal role such mechanisms perform in facilitating normal bacterial physiology, as well as the high conservation across bacterial pathogens that increases the range of species susceptible to a drug that targets these processes [6]. These include classes of drugs that inhibit cell wall biosynthesis, ribosomal activity, and DNA replication and repair [7]. Individual drugs within each drug class may differ in terms of their potency, cellular bioavailability, range of susceptible bacterial species, associated adverse reactions, and even in the kind of resistance mechanism most commonly attributed to the drug. The repertoire of available antibiotics is in the hundreds [8]. However, considering the likelihood of resistance and the needs of individual patients in terms of contraindications, the options for patients who harbor resistant pathogens shrink to a handful. It is important to note that resistance to the most widely used drug of last resort, vancomycin, is steadily increasing in frequency [9].

The crux of the antibiotic resistance epidemic we face today, in terms of its longevity and the speed of its spread, can be related to the fitness costs attributed to resistance. These costs are consistently among the most important factors that determine the rate and extent of the emergence of resistance [10–12]. The cellular machinery responsible for essential bacterial functions have evolved over millennia and while resistance mutations on these genes may allow for continued growth in the presence of an antibiotic, a variety of fitness costs often manifest when drug is withdrawn. These include reductions in growth rate or virulence, or in increased clearance rates [13]. It was assumed, therefore, that the absence of selection would disfavor the maintenance of costly resistance. This has clearly not been the case and is exemplified by the increasing rise in community-acquired resistant infections [14–16]. This troubling statistic suggests that resistance determinants are readily acquired in areas where drug use is limited, and the bacteria that carry these genes or mutations are able to compete effectively against environmental strains. A reevaluation of our understanding of antibiotic resistance and the fitness deficits this may entail is overdue.

**The problem of resistance from the perspective of bacteria undergoing antibiotic exposure**

In this dissertation, I argue that broadening the frame of study to include the responses of bacteria *during* antibiotic treatment can help explain different facets of the problem of antibiotic resistance. Specifically, I suggest that the presence of antibiotics is an important though currently overlooked context in which to measure bacterial fitness and document bacterial responses. In the first two research chapters, I address aspects that contribute to the magnitude and urgency of the problem of resistance. I provide evidence that suggests a role for antibiotic exposure in maintaining resistance as well as proposing a means in which resistant bacteria treated with antibiotics can be favored in the presence of a second, unrelated antibiotic. Last, I consider novel treatment strategies aimed at reducing the probability of resistance and demonstrate how an understanding of the responses of bacteria to antibiotics is a crucial first step in developing clinically effective and sustainable therapeutic regimes.

As a framework for the entire dissertation, here I review the mechanisms of resistance and resistance acquisition, how this relates to fitness costs, and importantly how environment affects fitness. Second, I examine facets of the problem of resistance that I contend are best approached from the perspective of bacteria as they endure and respond to antibiotic therapy. These aspects are then addressed in the subsequent research chapters.

**Mechanistic links between antibiotic resistance, fitness costs, and environmental context**

The first distinction that needs to be made is to separate resistance determinants into chromosomal mutations that alter the targets of antibiotics, and dedicated resistance genes that are usually carried by accessory elements. The second category primarily consists of enzymes that specifically degrade or modify antibiotics, or pumps that remove the antibiotic from the cell.

In Chapters 2 and 3, I employ *S. enterica* and *E. coli* bearing mutations on *rpsL*, which grants resistance to streptomycin. This drug binds to the 16S rRNA of the 30S ribosomal subunit and induces codon misreading [17]. Although the clinical utility of this antibiotic is limited due to the intravenous mode of delivery required, the variety of mutations and the associated costs and characteristics these mutations impart on bacteria has established streptomycin as a cornerstone of many laboratory studies. Numerous mutations on *rpsL,* the gene that encodes the highly conserved S12 protein of the ribosomal accuracy center, lead to resistance to streptomycin [18]. The diversity of effects of mutations on *rpsL* ranges from no costs on fitness, severe reductions in fitness *in vitro* and in mice, and even dependency on the presence of the drug for growth [19]. As a concrete illustration of the relationship between resistance and cost, in some cases the mechanism of resistance and the mechanism underlying the fitness cost are the same. The codon misreading produced by streptomycin can be corrected by so-called restrictive *rpsL* mutations; this sub-group of mutations increases the rate of proofreading and translational accuracy at the expense of decreasing protein elongation rates [20]. Irrespective of the mechanism of action of an antibiotic, the majority of studies report fitness costs associated to resistance caused by a mutation to the target of a drug [21].

While resistance determinants carried on extra-chromosomal elements such as plasmids do not involve changes to essential cellular machinery, a number of studies have similarly reported fitness costs associated with the carriage of plasmids [22]. The plasmid itself may result in a cost to the cell via the metabolic burden it imposes through it's maintenance and duplication during every cell division event [23]. The expression of dedicated resistance enzymes may impose some energetic cost, and efflux pumps may increase the permeability of the cell wall leading to the leakage of cellular components [24]. Currently however, it is not certain how common these costs are and how they may change across different combinations of plasmids and hosts. Furthermore, regulatory systems encoded in the plasmid may help limit the expression of these genes thus reducing costs in the absence of drug [25]. Finally, instead of ameliorating the costs of a resistance element carried by a plasmid to favor the maintenance of this trait, plasmids often have mechanisms that ensure their faithful transmission to each daughter cell during cell division [26]. As a consequence, the mechanisms that aid the transmission of plasmids indirectly aid the maintenance of the genes carried on the plasmid. In addition, the problem of

resistance is exacerbated by the ability of some plasmids to infect many different bacterial species. This could lead to the swift dissemination of resistance across a particular bacterial population, and even across diverse bacterial communities.

Regardless of the location of a resistance determinant, either on the chromosome or on an accessory element, an increasing number of studies highlight the importance of environment in exposing costs to resistance. The costs of norfloxacin resistance in *E. coli*, the most common causative agent of urinary tract infections, varies depending on whether fitness is measured *in vitro*, in urine, or in a mouse [27]. Furthermore, the relationship between environment and resistance may have unexpected effects besides modifying fitness costs due to resistance. Environmental changes may even facilitate the evolution of resistance, as when *E. coli* evolved in high temperatures but in the absence of any antibiotic consistently acquired resistance mutations to rifampin [28]. The opposite has also been observed with environment constraining the evolutionary trajectory of bacteria. While bacteria containing a costly fusidic acid resistance (FusR) mutation passaged in rich media were able to acquire secondary mutations that alleviated their fitness loss (see Chapter 2 for more on second-site compensatory mutations), this was not observed in the same bacteria passaged in mice. Mechanistically, this is believed to be caused by the altered levels of the nucleotides (p)ppGpp in FusR mutants [29]. These nucleotides are pleiotropic regulators of gene expression and changes in their concentration affect virulence genes, which in turn influences the fitness of FusR bacteria in mice [30]. Finally, the environmental context in which bacteria are observed has been shown to have clear clinical implications. Some types of colistin resistance in the hospital-acquired pathogen, *Acinetobacter baumanii*, only become detectable when the environmental growth conditions of the clinical assay are altered [31]. A range of growth conditions are necessary to not just accurately describe the costs of resistance but in some cases even reveal the presence of resistance.

In the following two chapters, my experiments employ chromosomal mutations for their strong and varied fitness costs. I take an under appreciated perspective and consider the effect of antibiotics on the fitness of *resistant* bacteria. This is an integration of two well-studied ideas: since resistance mutations are often costly and the costs of resistance can vary depending on environment, one would expect that an antibiotic-containing environment may affect the fitness or responses of bacteria resistant to that antibiotic. The results I present in the proceeding chapters demonstrate how this perspective adds to our understanding of resistance - how it is maintained, how it may extend in scope, and how the effects in the presence of an antibiotic may even be harnessed to inform the choices we make in the clinic.

**Maintenance of costly antibiotic resistance**

Although antibiotic resistance in human pathogens was first recognized shortly after antibiotics had been put to use in medical settings, there are still basic aspects of this problem that we do not fully understand [2]. This gap in our conception is exemplified in our insufficient attempts at devising strategies that can reverse the evolution of resistance or even control its spread. In seeking to achieve both of these goals, studies have focused on the disparity in fitness of resistant genotypes in the presence and absence of drug selection. Since antibiotic treatment would without question favor resistant over sensitive bacteria, the opposite scenario was similarly the expected outcome; a reduction in antibiotic usage was thought to favor replacement of resistant by sensitive bacteria [32]. The rise of resistant pathogens in areas where antibiotic use is limited has lead to an adjustment in our perception of the conditions in which resistance emerges and is maintained [14]. Studies aimed at explaining this result have focused on the genetic mechanisms by which bacteria reduce fitness costs due to resistance in order to remain competitive against sensitive bacteria, and more recently whether and how different environments may affect this competition [30,33]. One such important environmental factor is clearly the presence or absence of an antibiotic, but also the effect of varying antibiotic concentrations.

A direct test of the hypothesis that withdrawing an antibiotic would expose the fitness difference between sensitive and resistant bacteria and would thus negatively impact the maintenance of resistance was performed in an entire county in Sweden from 2004 until 2006 [34]. The folate synthesis inhibitor trimethoprim was voluntarily discontinued, replaced with an appropriate alternative, and resistance was monitored in *Escherichia coli* isolated from urinary tract infections. Although trimethoprim-containing drug use dropped by 85%, no appreciable effect on the frequency of resistance was reported. Laboratory experiments suggested that a lack of detectable costs as well as strong co-selection by other antibiotics were responsible for this disappointing result.

Although it is clear that therapeutic levels of drug quickly select for resistance, the role of low antibiotic concentrations that are present in many environments has only recently been studied. Using highly sensitive competition assays, Gullberg and colleagues demonstrate how selection for resistance could occur in antibiotics several hundred-fold below the minimal inhibitory concentration [35]. They show that while the deleterious effects on susceptible bacteria in very low antibiotic levels may be slight, the disparity this creates between the fitness of sensitive and resistant genotypes is nevertheless sufficient to favor resistant bacteria.

While the competition between resistant and sensitive bacteria can be affected by small changes in fitness induced by sub-inhibitory amounts of antibiotic, little attention has been paid to how antibiotics may influence competition among only resistant bacteria. Depending on the resistance determinants they

carry, these bacteria will express varying degrees of fitness, virulence, and susceptibility to antibiotics. Depending on their genetic background, resistant bacteria may even vary in their probability of reverting back to an antibiotic susceptible genotype.

In Chapter 2, my collaborators and I ask how antibiotic concentration and secondary mutations may affect the fitness of sensitive and resistant bacteria, and whether this interplay can predict the likelihood of reversion. The second site mutations we assess are a commonly recognized but poorly understood mechanism by which pathogens are thought to modulate costly resistance. Instead of losing the mutation responsible for resistance, numerous laboratory studies have shown that bacteria are more likely to acquire a second-site mutation that is only beneficial in the presence of the resistance mutation [36–40]. In this chapter, we probe the relationship between fitness and drug concentration by creating fitness landscapes of sensitive and resistant bacteria to determine whether combinations of drug levels and mutations may lead towards reversion.

**Genetic and environmental interactions that aid the spread of resistance**

The costs of resistance play a central role in determining whether resistance is maintained, spreads, or is lost [41,42]. The rise of multidrug resistant and even extensively drug resistant tuberculosis suggests that bacteria are able to modify these costs, or survive despite these costs [43,44]. Understanding the process by which bacteria acquire and retain not just one but many costly resistance elements is necessary in addressing and preventing the challenging infections we already face.

A specific resistance mutation may impose a range of costs dependent on the genomic context. These fitness effects may vary in strength or even form contingent on the presence of other mutations, including a second resistance allele. Using *Escherichia coli* transduced to carry pairs of different mutations, Trindade and colleagues performed competition assays in the absence of drug to measure epistasis between resistance determinants [45]. Besides the pervasive positive epistasis they find between resistance alleles with respect to fitness in the absence of drug, they also report that some cases of double resistance produce no measurable cost or are even beneficial when combined. Altering the deleterious effect of single resistance mutations with a second resistance allele provides a possible explanation for why multidrug resistance is so widespread and why its eradication is so difficult.

Aside from mutations, one study has implicated the environment in ameliorating the costs of deleterious mutations. Slow growing bacteria created via random mutagenesis were grown in the presence and absence of different stressors to determine whether such environments could reduce the average cost of a deleterious mutation [46]. Researchers used a variety of conditions

ranging from osmotic pressure to low temperature, including low concentrations of antibiotics. In contrast to the perception that carrying a deleterious mutation reduces an organism's tolerance to stress, they find certain environments that on average alleviate the cost of random deleterious mutations.

Medically important bacteria, both pathogens and commensals, navigate environments in which they are exposed to a large range of antibiotics and antibiotic concentrations. These include their time within a human host in which drug penetration and first-pass metabolism will create patches of low and high drug, and as they travel between hosts such as in hospitals where antibiotics are in abundant supply. In Chapter 3, I ask whether environments containing subinhibitory antibiotic concentrations may alleviate the cost of a resistance mutation to a second antibiotic. In the course of addressing this question, I find surprising results that indicate how growing resistant bacteria in the antibiotic to which it is resistant will affect its future success in a second drug. These findings have implications on how antibiotic pollution may promote the maintenance of costly resistance, and also induce a range of responses only available to resistant bacteria.

**Manipulating cellular responses to antibiotics**

Our nearly 100 years of experience using antibiotics in the clinic has shown repeatedly that pathogenic bacteria will evolve resistance to the drugs we discover or develop. This observation, however, has not changed the approach the scientific and medical communities advocate in tackling bacterial resistance. Although new molecules such as biofilm or virulence determinants are being recommended as targets for novel antimicrobials, history suggests that revitalizing the antibiotic pipeline is only a temporary solution [4,47,48]. The ease at which bacteria acquire, maintain, and even diversify their resistance arsenal demands a solution that combines novel drug discovery with sustainable drug policies that aim to delay the evolution of resistance and lengthen the useful life of our available antibiotics.

The gold standards in the treatment of the most widespread chronic infectious diseases of our period, HIV and tuberculosis, explicitly consider the prevention of resistance in their rationale [49,50]. In both examples, using combinations of drugs has been shown to effectively delay resistance. For these diseases, the chronic nature of the infection demands a sustained treatment duration. This increases the opportunity of the pathogen to evolve resistance. Most bacterial infections, however, are acute and drugs are prescribed for only a few days. Treatment failure due to resistance is not yet a concern in devising antibiotic dosing strategies.

Though there are a few cases of acute bacterial infections for which combination therapy is recommended, the underlying reasoning for their usage is to exploit a synergistic interaction between antibiotics [51]. These specific pairs

of drugs together have been proven to produce an effect greater than the combination of their individual effects. Aside from synergy, pairs of drugs may also interact in opposition with each other and such antagonistic interactions have been shown to result in treatment failure [52–54].

The two principles of resistance prevention or drug synergism that form the basis of combination therapy in chronic or acute infections are not in opposition to each other. The range of demonstrated benefits already attributed to combination therapy suggests that a reappraisal of this strategy towards the treatment of a larger number of bacterial infections has the potential to both effectively treat patients as well as delay the emergence of resistance.

As a first step towards the wider usage of combination therapy, in Chapter 4 I propose a simple explanation for when certain antibiotic pairs would result in an antagonistic interaction. I hypothesize that a basic pharmacodynamic property of all drugs is a useful aid in predicting when a specific combination will result in antagonism. Since the killing effect of bactericidal drugs is potentiated by active bacterial growth, we rationalize that these drugs would be antagonized by the simultaneous application of an antibiotic that induces cell stasis. We perform a screen of pairwise interactions among more than 20 different antibiotics and find that a vast majority of antagonistic pairs are composed of a bactericidal and a bacteriostatic drug. We confirm these findings for a subset of these combinations on the population level using time kill curves and on the single cell level using a microfluidic device. We provide direct visual and quantitative information on how the induction of cellular stasis leads to the survival of a population exposed to inhibitory drug concentrations. Our results demonstrate how observations of bacterial responses to drugs could aid in the design of new treatment strategies.

**Insight into the problem of resistance from the perspective of bacteria exposed to antibiotics**

Determining the resistance mechanisms of pathogens has lead to tangible changes in how bacterial infections are treated. Among the oldest of the drugs still in use today, antibiotics of the beta-lactam class are still valuable due to such work. One of the most common mechanisms of resistance bacteria have evolved against beta-lactams is beta-lactamase [7]. This enzyme directly degrades members of the penicillin, cephamycin, and carbapenem families. These antibiotics remain effective, however, due to the development and inclusion of beta-lactamase inhibitors in the same drug preparation as their beta-lactam partners [55]. The discovery of beta-lactamase inhibitors also guided the design of methicillin, a penicillin antibiotic insensitive to penicillinase. Unsurprisingly, the continued utility of these drugs has come into question due to the emergence of resistance to beta-lactamase inhibitors and to methicillin [56].

Pathogenic bacteria have consistently and quickly evolved resistance to every drug employed in clinics, suggesting that novel drug design alone is only a shortsighted response to the problem of antibiotic resistance. Although this strategy has not proven to be sustainable, biomedical researchers and economists are clamoring for changes in policies from governments to address the problem of resistance by revitalizing the antibiotic pipeline [57,58]. They argue that a combination of tax incentives and advance purchase contracts would sustain the research and production of antibiotics to maintain our lead in treating patients with resistant infections. It is not disputed that new drugs are sorely needed, however this solution alone would only strengthen our dependence on continuous drug development. The widening scope and increasing incidence of resistant infections suggests that the manner in which antibiotics are prescribed must be reassessed to safeguard the needs of patients, slow the evolution of resistance, and extend the useful life of the drugs we have.

In this dissertation, I argue that broadening the focus of study to include how both sensitive and resistant bacteria respond to a range of antibiotic concentrations will provide new insights into an established, enduring, and increasing problem. As resistance has spread from the hospital to include the community, our concept of when and how resistance emerges and is maintained must be similarly readjusted. In order to maintain our lead over constantly evolving pathogens, solutions to the problem of antibiotic resistance cannot rely solely on the development of new drugs. Close attention must be paid on how and to what extent antibiotics are deployed in treatment and in the environment to delay the spread of resistance and preserve our ability to treat bacterial infections.

**References**


1. Demain AL, Sanchez S (2009) Microbial drug discovery: 80 years of progress. J Antibioti 62: 5–16. doi:10.1038/ja.2008.16.
2. Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: A new paradigm for antimicrobial therapy. Nat Chem Biol 3: 541–548.
3. Cegielski JP (2010) Extensively drug-resistant tuberculosis: "there must be some kind of way out of here". Clin Infect Dis 50 Suppl 3: S195–200. doi:10.1086/651491.
4. Fischbach M a., Walsh CT (2009) Antibiotics for Emerging Pathogens. Science 325: 1089–1093. doi:10.1126/science.1176667.
5. D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. Science 311: 374–377. doi:10.1126/science.1120800.
6. Becker D, Selbach M, Rollenhagen C, Ballmaier M, Meyer TF, et al. (2006) Robust Salmonella metabolism limits possibilities for new antimicrobials. Nature 440: 303–307. doi:10.1038/nature04616.
7. Walsh C (2000) Molecular mechanisms that confer antibacterial drug resistance. Nature 406: 775–781. doi:10.1038/35021219.
8. Gilbert D, Moellering R, Eliopoulos G, Chambers HF, Saag M, editors (2010) The Sanford Guide to Antimicrobial Therapy. 40th Ed. Sperryville, VA: Antimicrobial Therapy, Inc.
9. Mckenna M (2013) Antibiotic Resistance: The Last Resort. Nature 499: 364–366.
10. Böttger E, Springer B, Pletschette M, Sander P (1998) Fitness of antibiotic-resistant microorganisms and compensatory mutations. Nature Medicine 4: 11–12.
11. Andersson DI (2003) Persistence of antibiotic resistant bacteria, Curr Opin Microbiol 6: 452–456.
12. Handel A, Regoes RR, Antia R (2006) The role of compensatory mutations in the emergence of drug resistance. PLoS Comput Biol 2: e137. doi:10.1371/journal.pcbi.0020137.
13. Beceiro A, Tomás M, Bou G (2013) Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev 26: 185–230. doi:10.1128/CMR.00059-12.

14. Klein E, Smith DL, Laxminarayan R (2009) Community-associated methicillin-resistant Staphylococcus aureus in outpatients, United States, 1999-2006. Emerg Infect Dis 15: 1925–1930. doi:10.3201/eid1512.081341.

15. Cunha BA (2004) Therapeutic implications of antibacterial resistance in community-acquired respiratory tract infections in children. Infection 32: 98–108. doi:10.1007/s15010-004-3065-5.

16. Fridkin S, Hageman J, Morrison M, Thomson Sanza L, Como-Sabetti K, et al. (2005) Methicillin-resistant Staphylococcus aureus disease in three communities. N Engl J Med 352: 1436–1444.

17. Hurwitz C (1963) Mechanism of action of streptomycin. Antimicrob Agents Chemother 161: 371–386.

18. Kurland CG (1992) Translational accuracy and the fitness of bacteria. Annu Rev Genet 26: 29–50. doi:10.1146/annurev.ge.26.120192.000333.

19. Bjorkman J, Samuelsson P, Andersson D, Hughes D (1999) Novel ribosomal mutations affecting translational accuracy, antibiotic resistance and virulence of Salmonella typhimurium. Mol Microbiol 31: 53–58.

20. Agarwal D, Gregory ST, O'Connor M (2011) Error-prone and error-restrictive mutations affecting ribosomal protein S12. J Mol Biol 410: 1–9. doi:10.1016/j.jmb.2011.04.068.

21. Andersson DI, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2: 489–493.

22. Wiedenbeck J, Cohan FM (2011) Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiol Rev 35: 957–976. doi:10.1111/j.1574-6976.2011.00292.x.

23. Sengupta M, Austin S (2011) Prevalence and Significance of Plasmid Maintenance Functions in the Virulence Plasmids of Pathogenic Bacteria. Infect Immun 79: 2502–2509. doi:10.1128/IAI.00127-11.

24. Allahverdiyev A, Kon K, Abamor E, Bagirova M, Rafailovich M (2011) Coping with antibiotic resistance: combining nanoparticles with antibiotics and other antimicrobial agents. Expert Rev Anti Infect 9: 1035–1052.

25. Bahl M, Hansen L, Sorensen S (2009) Persistence Mechanisms of Conjugative Plasmids. Methods Mol Biol. pp. 73–102.

26. Ebersbach G, Gerdes K (2005) Plasmid segregation mechanisms. Annu Rev Genet 39: 453–479. doi:10.1146/annurev.genet.38.072902.091252.

27. Lindgren PK, Marcusson LL, Sandvang D, Frimodt-møller N, Hughes D (2005) Biological Cost of Single and Multiple Norfloxacin Resistance Mutations in Escherichia coli Implicated in Urinary Tract Infections. Antimicrob Agents Chemother 49: 2343–2351. doi:10.1128/AAC.49.6.2343.

28. Rodríguez-Verdugo A, Gaut BS, Tenaillon O (2013) Evolution of Escherichia coli rifampicin resistance in an antibiotic-free environment during thermal stress. BMC Evol Biol 13: 50. doi:10.1186/1471-2148-13-50.

29. Macvanin M, Johanson U, Ehrenberg M, Hughes D (2000) Fusidic acid-resistant EF-G perturbs the accumulation of ppGpp. Mol Microbiol 37: 98–107.

30. Björkman J, Nagaev I, Berg OG, Hughes D, Andersson DI (2000) Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance. Science 287: 1479–1482. doi:10.1126/science.287.5457.1479.

31. Snitkin ES, Zelazny A, Gupta J, Palmore TN, Murray PR, et al. (2013) Genomic insights into the fate of colistin resistance and Acinetobacter baumannii during patient treatment. Genome Res. doi:10.1101/gr.154328.112.

32. Lenski R (1997) The cost of antibiotic resistance - from the perspective of a bacterium. Ciba Found Symposium 207: discussion 141–51.

33. Andersson DI (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol 9: 461–465. doi:10.1016/j.mib.2006.07.002.

34. Runehagen A, Cars H, Sundqvist M, Geli P, Andersson DI, et al. (2010) Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. J Antimicrob Chemother: 350 – 360. doi:10.1093/jac/dkp387.

35. Gullberg E, Cao S, Berg O, Ilbäck C (2011) Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 7: 1–9. doi:10.1371/journal.ppat.1002158.

36. Lind P a, Tobin C, Berg OG, Kurland CG, Andersson DI (2010) Compensatory gene amplification restores fitness after inter-species gene replacements. Mol Microbiol: 1–12. doi:10.1111/j.1365-2958.2009.07030.x.

37. Reynolds M (2000) Compensatory evolution in rifampin-resistant Escherichia coli. Genetics 156: 1471–1481.

38. Nagaev I, Björkman J, Andersson DI, Hughes D (2001) Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. Mol Microbiol 40: 433–439.

39. Cortes PR, Cian MB, Albarracı AG (2011) Compensatory Evolution of pbp Mutations Restores the Fitness Cost Imposed by b-Lactam Resistance in Streptococcus pneumoniae. PLoS Pathog 7: e1002000. doi:10.1371/journal.ppat.1002000.

40. Paulander W, Maisnier-Patin S, Andersson DI (2007) Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in Salmonella typhimurium. Mol Microbiol 64: 1038–1048. doi:10.1111/j.1365-2958.2007.05713.x.

41. Bonhoeffer S, Lipsitch M, Levin BR (1997) Evaluating treatment protocols to prevent antibiotic resistance. Proc Nat Acad USA 94: 12106–12111.

42. Lipsitch M, Levin BR (1997) The population dynamics of antimicrobial chemotherapy. Microbiology 41(2):363.

43. Cox HS, Niemann S, Ismailov G, Doshetov D, Orozco JD, et al. (2007) Risk of acquired drug resistance during short-course directly observed treatment of tuberculosis in an area with high levels of drug resistance. Clin Infect Dis 44: 1421–1427. doi:10.1086/517536.

44. Seung KJ, Gelmanova IE, Peremitin GG, Golubchikova VT, Pavlova VE, et al. (2004) The effect of initial drug resistance on treatment response and acquired drug resistance during standardized short-course chemotherapy for tuberculosis. Clin Infect Dis 39: 1321–1328. doi:10.1086/425005.

45. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, et al. (2009) Positive epistasis drives the acquisition of multidrug resistance. PLoS Genet 5: e1000578. doi:10.1371/journal.pgen.1000578.

46. Kishony R, Leibler S (2003) Environmental stresses can alleviate the average deleterious effect of mutations. J Biol: 1–10.

47. Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Dis 9: 117–128. doi:10.1038/nrd3013.

48. Kalan L, Wright GD (2011) Antibiotic adjuvants: multicomponent anti-infective strategies. Expert Rev Mol Med 13: 1–17. doi:10.1017/S1462399410001766.

49. Hand K (2006) Tuberculosis: Pharmacological Management. Hospital Pharmacist 13: 81–85.

50. Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, et al. (1998) Changing patterns of mortality across Europe in patients infected with HIV-1. Lancet 352: 1725–1730.

51. Fischbach MA (2011) Combination therapies for combating antimicrobial resistance. Curr Opin Microbiol 14: 519–523. doi:10.1016/j.mib.2011.08.003.

52. Johansen HK, Jensen TG, Dessau RB, Lundgren B, Frimodt-Moller N (2000) Antagonism between penicillin and erythromycin against Streptococcus pneumoniae in vitro and in vivo. J Antimicrob Chemother 46: 973–980.

53. Cates J, Christie R, Garrod L (1951) Penicillin-resistant Bacterial Endocarditis Treated by Penicillin and Streptomycin. Brit Med J 1: 656–656.

54. Garrod L (1972) Causes Of Failure In Antibiotic Treatment Garr. Brit Med J 4: 473–476.

55. Saudagar PS, Survase SA, Singhal RS (2008) Clavulanic acid: a review. Biotechnology advances 26: 335–351. doi:10.1016/j.biotechadv.2008.03.002.

56. Mostofsky E, Lipsitch M, Regev-Yochay G (2011) Is methicillin-resistant Staphylococcus aureus replacing methicillin-susceptible S . aureus? J Antimicrob Chemother 66: 2199–2214. doi:10.1093/jac/dkr278.

57. Coast J, Smith RD, Millar MR (1998) An economic perspective on policy to reduce antimicrobial resistance. Social science & medicine (1982) 46: 29–38.

58. Nathan C, Goldberg FM (2005) The profit problem in antibiotic R&D. Nat Rev Drug Dis 4: 887–891.

# 2

# The fitness landscapes of an antibiotic resistance mutation and its associated compensatory mutations

**Paolo S. Ocampo[1,5], Jan Engelstädter[2], Pia Abel zur Wiesch[3], Martin Ackermann[4,5], Sebastian Bonhoeffer[1]**

[1]Institute of Integrative Biology, ETH Zurich
[2]School of Biological Sciences, University of Queensland
[3]Department of Global Health and Social Medicine, Harvard Medical School
[4]Institute of Biogeochemistry and Pollution Dynamics, ETH Zurich
[5]Department of Environmental Microbiology, EAWAG

# Abstract

The increasing incidence of resistant infections suggests that bacteria are able to maintain their often costly resistance mutations even when antibiotic usage is low. Understanding the mechanisms by which bacteria reduce these fitness costs is a crucial first step in devising strategies to slow the progression and address the possibility of reversing the evolution of resistance. The acquisition of a compensatory mutation, a second-site mutation that is beneficial in the presence of a resistance mutation but is otherwise deleterious, is one means in which bacteria are thought to alleviate the cost of resistance and to disfavor reversion to a sensitive state. Our current understanding of the effects of these mutations is hampered by a lack of quantitative observations. In this study, we sought to clarify the influence of compensatory mutations in maintaining costly resistance by obtaining drug-dependent fitness landscapes involving different compensatory mutations. We employed a set of *S. enterica* serovar *typhimurium* strains consisting of one sensitive and one streptomycin resistant genotype, 17 variants of the resistant strain each bearing one specific compensatory mutation, and the corresponding sensitive genotypes each bearing the same compensatory mutations. The measurements of growth and death rates we performed across a large range of antibiotic concentrations described landscapes that are characterized by a strong dependence on antibiotic concentration. We find that the relative fitness of compensatory mutations in a susceptible and resistant strain background strongly depends on drug concentration. For some concentrations, compensatory mutations increase the fitness of both sensitive and resistant bacteria. We drew on these results to model the evolutionary dynamics of these genotypes as they undergo antibiotic treatment. The range of effects we observed across different alleles in different drug concentrations led to scenarios in which both resistant and sensitive genotypes co-occur.

# Introduction

The problem of antibiotic resistance is characterized not only by the ease at which resistance determinants are acquired, or the frequency at which multi-drug resistance is observed, but also by the duration in which resistance persists after drug use is discontinued. The troubling rise in resistant infections suggests that there are gaps in our understanding of antibiotic resistance [1–3]. The acquisition of resistance is often associated with fitness costs to the bacteria; these can manifest as reductions in growth rate or virulence, or increased rates of clearance of the infection [4–6]. This disparity in fitness between resistant and sensitive bacteria predicts that the discontinuation of a drug would cause a reduction in the incidence of resistant infections. Numerous experimental studies testing this hypothesis instead observed the acquisition of compensatory mutations, second-site mutations that alleviates the cost of resistance but are deleterious on their own [7–12].Compensatory mutations may create a local fitness peak with a fitness valley between the compensated resistant and the sensitive genotype, which is then assumed to prevent or slow down reversion to susceptibility in the absence of drug.

In order to properly assess the influence of compensatory mutations in maintaining costly resistance, it is first required to determine the conditions under which such fitness peaks are created. Only one laboratory study has measured the effects of these mutations in both the presence and absence of drug [13]. They find that compensatory mutations selected when treatment is withdrawn only increase fitness in a drug-free environment. Furthermore, although specific compensatory mutations to an assortment of antibiotic resistance mutations have been identified, no study has measured the fitness effects of compensatory mutations in a sensitive strain background.

Obtaining such information may help explain the divergent clinical results – while laboratory experiments consistently cite compensatory mutations as barriers to reversion, evidence from patients is inconsistent and inconclusive regarding the influence of these mutations [12]. Reversion may also be more favorable *in vivo* than *in vitro* as indicated by a number of reports from individual patients [4,14–16].

Recently, theoretical studies have sought to reconcile the contrasting reports from the lab bench and from patients. Attempts at bridging this gap have considered the possibility of differential fitness effects in the presence and absence of drug, or the effect of fluctuating environments on determining reversibility [17,18]. Compensatory mutations have the potential to strongly influence the evolution and maintenance of resistance. A more complete grasp of their fitness effects is required to accurately assess their importance and devise strategies to prevent their acquisition.

In this study, we contribute to the understanding of compensatory mutations by measuring the fitness effects as growth or death rates of all the relevant genotypes across a wide antibiotic concentration range. The set of strains we employ are described in [9]. In brief, the study in which the strains were produced sought to determine the diversity of mutations that can compensate for a specific resistance mutation to streptomycin. They considered the K42N substitution on *rpsL*, which increases the rate of ribosomal proofreading leading to decreased bacterial growth and decreased virulence in *Salmonella enterica* serovar *typhimurium*. They serially passaged 81 independent lines of this mutant in the absence of antibiotic until fitness was observed to increase. Four lines were found to have regained fitness by losing the resistance mutation, however the 77 other lines remained streptomycin resistant and contained at least 35 different amino acid substitutions that did not appear during passage of sensitive control lines. These 35 second-site mutations as well as the original resistance mutation were separated and then combined again using P22-mediated transduction. The results we present are based on half of the set of strains they constructed.

Our study population consists of the sensitive *S. enterica* serovar *typhimurium* strain (denoted ++) and the streptomycin resistant version (R+), 17 resistant strains each bearing a specific compensatory mutation (RC), and the 17 corresponding sensitive strains (+C). We use our estimates of growth or death rates to describe fitness landscapes in the absence of drug and then extend our exploration from very low to high drug concentrations to determine the effects these environments may have in shaping a genotype's fitness. We apply our experimental results to simulations of the evolutionary dynamics of these strains as antibiotic therapy is applied and subsequently removed. In this fashion, our model determines the effect of compensatory mutations on the maintenance of costly antibiotic resistance and the likelihood of reversion to a sensitive genotype.

# Results

### Increasing drug concentration produces diverse effects on fitness landscapes

Figure 1 depicts three plots, each showing the fitness measurements of a different pair of RC and +C strains along with the ancestral sensitive and resistant (++ and R+) across the entire range of antibiotic concentration considered in this study. Supplementary Figure 1 presents the entire set of dose-response curves.

The dose-response curves we obtained are highly heterogeneous and are strongly dependent on antibiotic concentration. There are conditions in which sensitive strains become less fit as drug increases, however the converse response is also evident - in all strains, across both sensitive and resistant geno-

types, we find areas of drug concentration in which growth rate increases as antibiotic dosage also increases. An example of this is illustrated in the middle panel of Figure 1, where the red line represents the fitness estimates for the RC strain bearing the C mutation, *rpsE* T130I. As streptomycin concentration increases from 5 to 10 ug/mL, the fitness of this strain increases significantly (unpaired, two-tailed t-test assuming unequal variance, $p = 0.0004$). However a further increase in concentration from 10 to 15 ug/mL results in a significant decrease in fitness (unpaired, two-tailed t-test assuming unequal variance, $p = 0.0001$).

These effects are also allele specific as different compensatory mutations in the same locus can produce significantly different growth or death measurements. As an example, consider an RC strain that contains either the *rpsD* N84D or N84S compensatory mutations (Refer to panels Compensatory Allele #3 and Compensatory Allele #4 in Supplementary Figure 1). The fitness of these two RC strains do not differ significantly at 1000 ug/mL, however raising the drug concentration to 10,000 ug/mL results in a significant drop in fitness of the N84S strain in comparison to N84D (unpaired, two-tailed t-test assuming unequal variance, $p < 10^{-8}$).

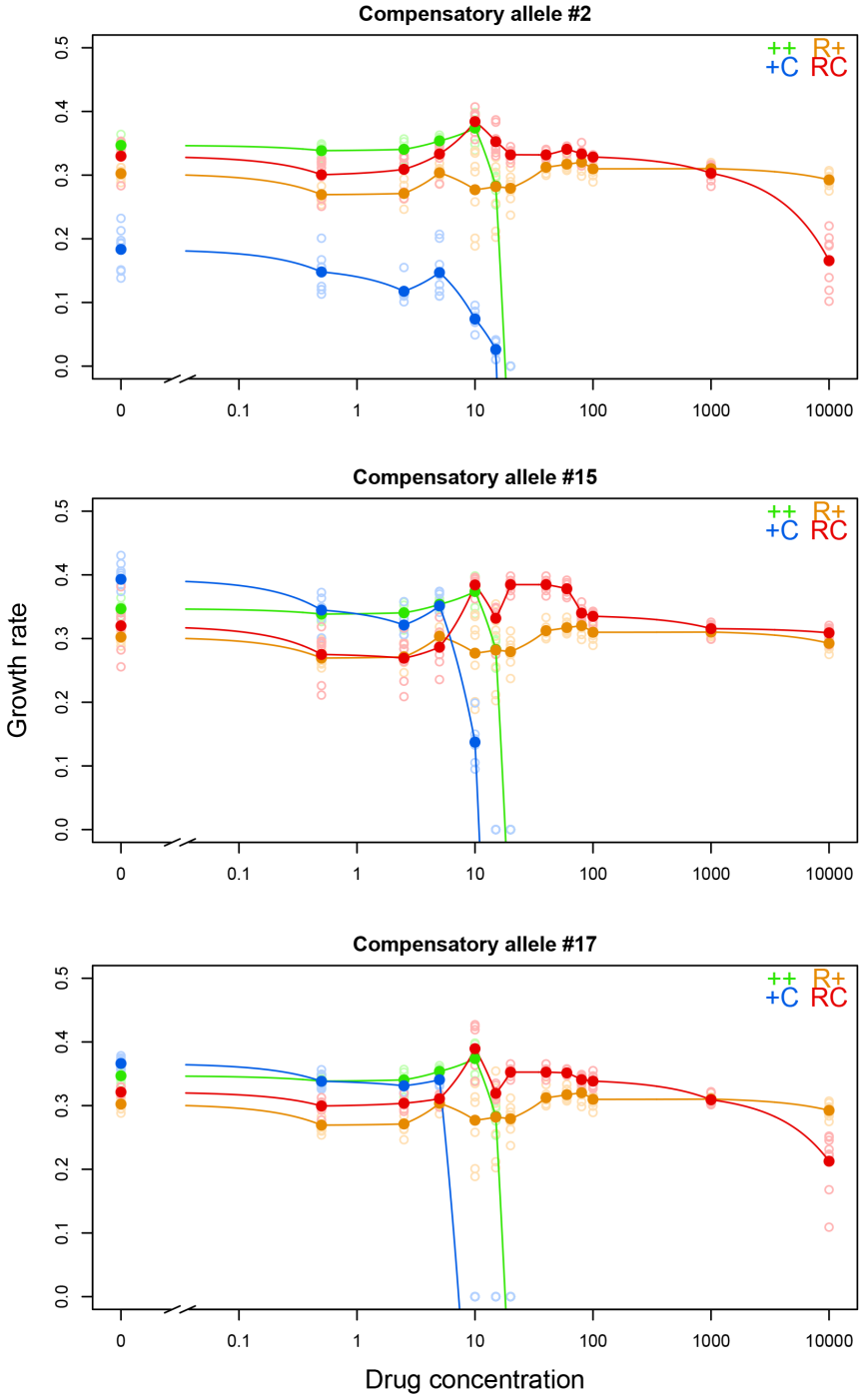



**Figure 1. Dose-response curves of three sets of streptomycin-sensitive (++), streptomycin-resistant (R+), resistant and compensated (RC), and the corresponding sensitive and compensated genotypes (+C).**

These plots depict the growth rates of a set of four genotypes across a range of streptomycin drug concentrations. To allow for a clearer comparison of the genotypes, the ancestral sensitive and resistant strains are shown along with the fitness effects of sensitive and resistant variants bearing compensatory allele #2, allele #15, and allele #17 in the lowest graph. Closed circles denote the mean of eight replicates (open circles).

As the original experimental conditions selected for mutations to emerge in a resistant strain in the absence of drug, we observed all RC strains to have higher fitness than R+ when there is no drug present. However, we found that the presence of these compensatory mutations can either increase or decrease the fitness of a resistant strain in the presence of drug. As an overview, Figure 2A plots the relative fitness differences between R+ and RC strains across every drug concentration measured. The boxplots above the 0 line denote the fitness advantage of RC over R+ strains as it extends across the range of drug concentrations measured, until the highest drug concentration where this situation reverses and RC strains had significantly lower growth rates than R+ (unpaired, two-tailed t-test assuming unequal variance, $p < 10^{-10}$).

The effects of the C mutations on the fitness of a sensitive strain span a wider range. In the absence of antibiotic and of the resistance mutation, some second-site mutations are deleterious while others displayed little to no cost, or even increase fitness. Interestingly, these effects segregate according to the gene of the C mutation. +C strains bearing mutations on *rpsD* were significantly less fit than ++ in the absence of drug (unpaired, two-tailed t-test assuming unequal variance, $p < 10^{-5}$), while C mutations on *rpsE* or *rplS* did not significantly affect fitness compared to the ancestral sensitive strain.

Our measurements of the effects of compensatory mutations on sensitive and resistant genomic backgrounds confirms that the combination of effects of mutation and dosage results in an RC strain being the most fit for a wide range of drug concentrations. The diversity of the effects we observed, however, raises the possibility that specific dosage and mutation combinations can disfavor the maintenance of resistance. This relationship between concentration and fitness may affect the widely acknowledged role of compensatory mutations in maintaining resistance in the absence of selection. Bergstrom and Feldgarden have described this effect as an "evolutionary lobster trap" [19]. They defined this process as a genotype easily accessible under selection in one direction, but difficult to leave when selection goes in the other direction. In the case of antibiotic resistance, a bacterial strain enters this trap if it acquires a compensatory mutation that is beneficial in the resistant strain background but is deleterious without the resistance allele.

Figure 2B shows how dosage can influence these fitness effects as the distribution of the relative magnitude of the lobster trap created by the compensatory mutations. Specifically, this graph plots the distribution of the relative fitness differences between each RC against the most fit sensitive partner, either the ++ or the corresponding +C. Positive values denote the beneficial effect of the compensatory mutation in a resistant background and the presence of a lobster trap situation – under that particular condition, reversion to sensitivity is disfavored. Negative values, however, suggest the opposite and this is ob-

served at very high antibiotic concentrations, as well as for some cases at sub-inhibitory concentrations.

Finally, Figure 2C extracts from each set of four strains (i.e., 17 sets of ++, R+, RC, and +C, differing by the 17 specific C mutations) which genotype is fittest at each concentration. The mixture of R+ and RC at high concentrations and ++, +C, and even RC in the absence of drug reiterates the range of fitness effects imparted by compensatory mutations originally selected in the absence of drug.

**Whole Genome Sequencing**

In order to corroborate the unexpected experimental results we obtained, whole genome sequencing was performed on the ++ and four +C strains to determine whether the fitness effects we observed could be due to other genetic changes aside from the introduced C mutations. The sequencing results revealed that the ++ and +C strains only differ by their respective C mutation.

**Model results strongly depend on drug concentration**

We applied our fitness measurements to model the *in vivo* dynamics of a bacterial population subjected to five days of streptomycin therapy at a clinically relevant concentration, followed by three months without antibiotics (Figure 3A). We were interested in determining whether sensitive genotypes could be detected under such conditions. We modeled 17 scenarios separately and allow for mutations, both resistance and a single compensatory mutation, to evolve and affect the population dynamics of the four emerging genotypes. Figure 3B depicts the population dynamics we observed for strains permitted to acquire allele #17. Finally, Figure 3C presents the average composition of the bacterial population for all 17 scenarios after the treatment and withdrawal periods.

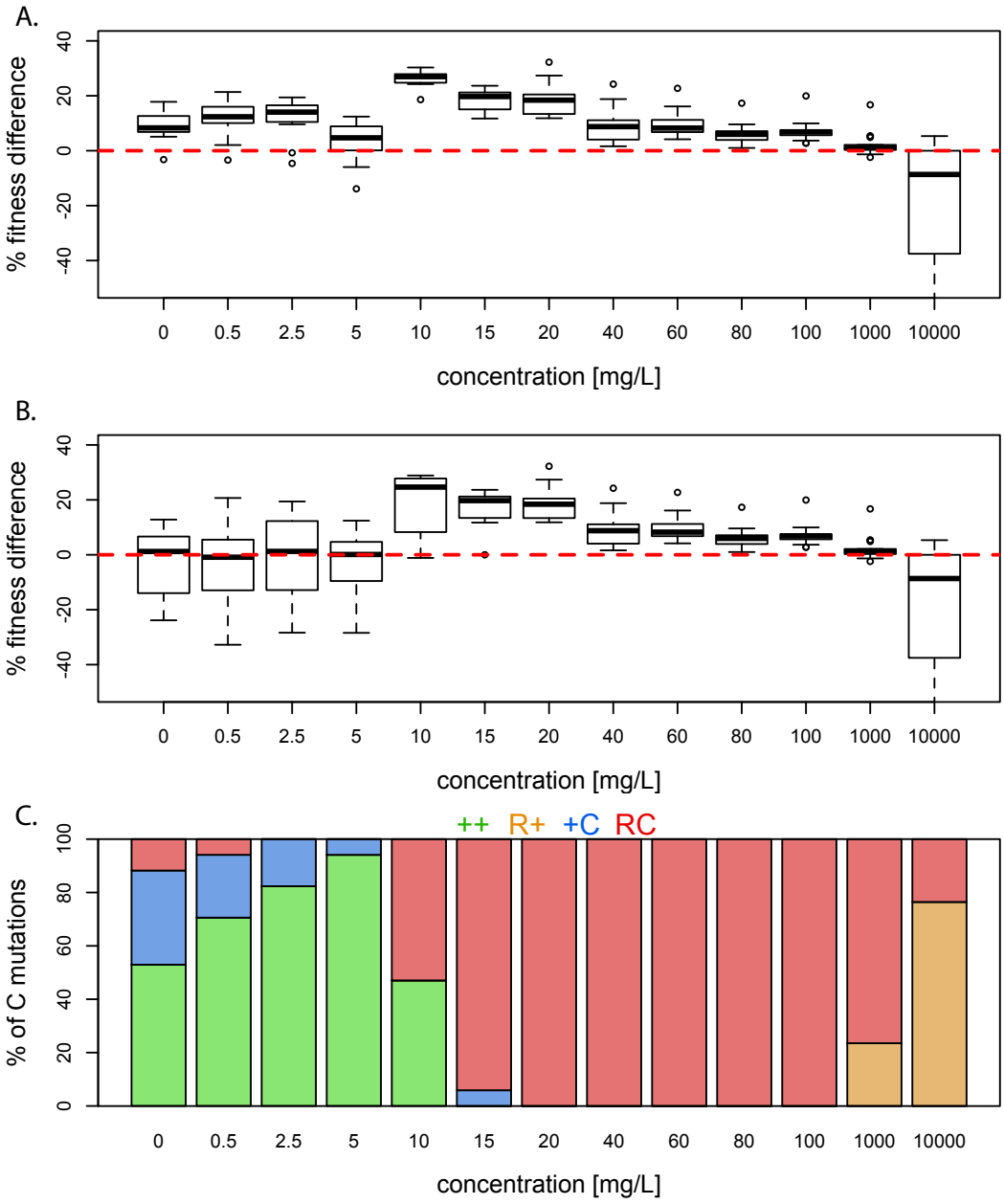



**Figure 2. Distributions of relative fitness effects of compensatory mutations.**

A. The distribution of relative fitness differences between R+ and RC ((wRC – wR)/wRC) across concentrations indicates the antibiotic conditions in which bearing a compensatory mutation is beneficial (positive values) and in which compensatory mutations incur fitness costs.

B. Compensatory mutations may "trap" the resistant genotype on a fitness peak. This distribution of the relative magnitude of this trap ((wRC – max(wR+,w+C))/wRC) reveals the conditions in which such a situation is present (positive values).

C. Plotting the proportions of the four genotypes in increasing drug concentration according to their fitness reveals the heterogeneous effects of compensatory mutations – some can be beneficial in the absence of drug and the majority are deleterious at high drug concentrations.

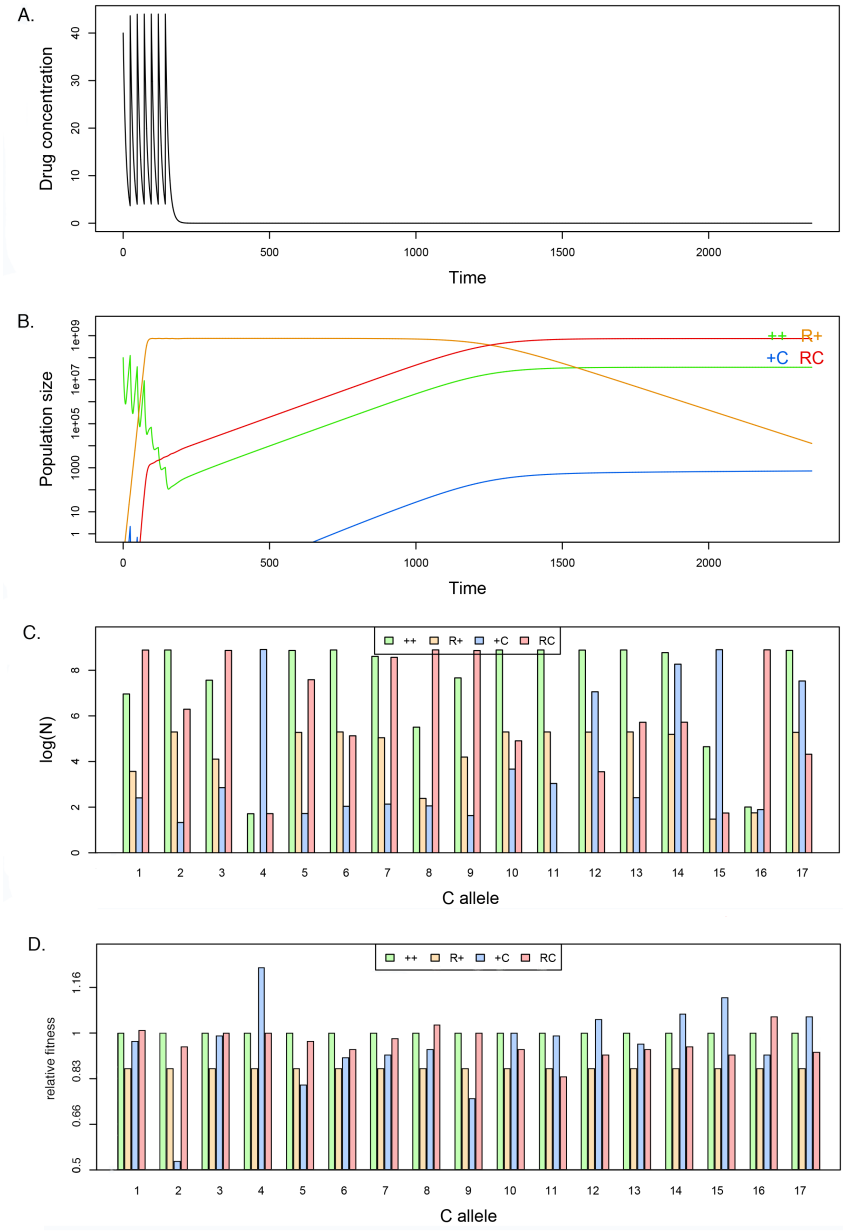



**Figure 3. Model Results**

A. Pharmacodynamics of the streptomycin treatment regime applied in the model. The sensitive ++ strain is exposed to a pulse of streptomycin reaching a maximum concentration of 40 ug/mL for 7 days, during which the resistance mutation as well as one specific compensatory mutation may evolve. Drug is then withdrawn and the emerging populations compete and are observed for an additional three months. This simulation is performed for each of the 17 different sets of RC, +C pairs.

B. Population dynamics of strains allowed to acquire compensatory allele #17.

C. Average composition of strain types for each of the 17 sets of four genotypes at the end of the simulation.

D. To facilitate comparison, the relative fitness of all the genotypes to the ++ in the absence of drug is presented.

**Simulations suggest compensatory mutations aid in the co-occurrence of resistant and sensitive genotypes.**

Our simulations provide evidence for a role of compensatory mutations in maintaining costly resistance in the absence of antibiotic selection – as well as favoring the reemergence of sensitive genotypes. Although our theoretical study yielded a wide variety of outcomes, the co-occurrence of sensitive and resistant bacteria was consistent for all 17 compensatory mutations.

The variety in the results we observed is striking and some results in particular stand out. For example, though resistant genotypes are the most abundant in every situation, this is not always the RC strain – for some cases, the R+ strain is present at equal or even higher proportions. Similarly, while sensitive genotypes are always present at the end of the observation period, there are cases in which the +C comprises a larger fraction of the population than the ++.

**Simulations underline the importance of fitness measurements performed across drug concentrations**

We observed that integrating the fitness measurements across a wide range of drug concentrations had profound effects on the population dynamics of resistant and sensitive bacteria in our simulation. Without these fitness landscapes, predictions would be made relying solely on fitness data in the absence of drug (Figure 3D). Comparing Figures 3C and 3D, we found cases in which the fitness measurements could not be used to estimate the simulation results. For example, the +C and RC strains of allele #3 display similar fitness measurements in the absence of drug (Figure 3D). In contrast, our model predicts that the RC would outcompete the +C (Figure 3C).

We next focused on the effect of antibiotic decay in influencing the results of the model. The pharmacokinetics of an antibiotic are an important factor in determining appropriate dosage strategies to achieve therapeutic concentrations for a sufficient duration to clear the infection, while avoiding the adverse side effects related to excessive drug levels [20]. Our model accounts for the gradual elimination of streptomycin that would be mediated by the kidneys during treatment. The fitness measurements along these decreasing drug concentrations may also affect the competition of resistant and sensitive bacteria. To illustrate the effect of decreasing concentration, Figure 4 considers two different pharmacodynamic scenarios. In the top panel of Figure 4A, we restrict the previously applied observation period to only seven days of daily streptomycin treatment reaching a maximum of 40 ug/mL but then decays. Without the data on fitness measurements at intermediate drug concentrations, one would effectively simulate the effect of a single, continuous dosage (Figure 4B). Aside from the quantitative differences between the two situations, there are obvious qualitative disparities: the middle plot in Figure 4A displays the reemergence of the drug-sensitive +C strain as the antibiotic concentration

drops during the last day of treatment. Furthermore, information on fitness across drug concentrations predicts the reemergence of sensitive bacteria in 5/17 cases while the other scenario does not (bottom plots in Figure 4).

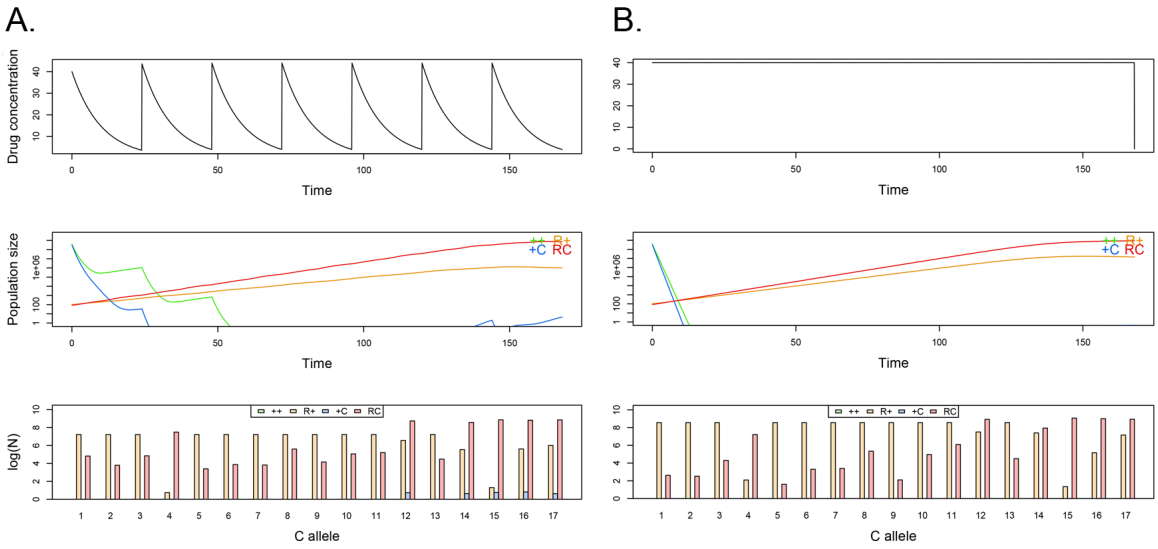



**Figure 4. Fitness across drug concentrations strongly affect the model**

The top plots in this figure show the pharmacodynamics of the streptomycin treatment regimes applied in the simulation. The middle plots depict the resulting population dynamics of a strain allowed to acquire compensatory allele #17. Finally, the bottom plots display the average composition of genotypes for each of the 17 sets of four genotypes at the end of the simulation

A) Knowledge of the fitness landscapes of sensitive and resistant genotypes allows for the consideration of intermediate drug concentrations during treatment on the population dynamics of sensitive and resistant bacteria. Sensitive bacteria are found to reemerge at low antibiotic levels in 5/17 conditions.

B) Ignoring the effect of varying dosage on fitness produces qualitatively different results – exemplified by the absence of any sensitive genotypes in every scenario.

# Discussion

**The rugged fitness landscapes of a resistance mutation and its compensatory mutation partners could help reconcile conflicting clinical and laboratory studies.**

Our results suggest that mutations acquired to alleviate costly antibiotic resistance can both avert or aid reversion to a sensitive genotype contingent on both the specific allele and antibiotic concentration. Two primary features of the fitness landscapes we produced could aid in explaining the diverse array of findings across clinical and laboratory studies: First, increasing antibiotic concentration produces non-monotonic changes in the fitness of both sensitive and resistant bacteria. Second, a significant proportion of compensatory mutations

are neutral or beneficial in a resistant strain background even in the absence of the resistance mutation.

Importantly, we find that the fitness of *all* four genotypes is strongly dependent on antibiotic concentration. For all four genotypes, we find areas of the dose-response curve in which increasing drug concentration can either lower or raise fitness. This latter case is known as the Eagle effect: Eagle and Musselman first recognized that the dose-response curve for some antibiotics is not completely monotonic [21]. We observe this phenomenon in some of the sensitive genotypes (++ and some of the +Cs). However the degree of this effect and the antibiotic concentrations under which it is most evident vary across alleles. These results suggest that some specific sub-inhibitory drug concentrations may paradoxically favor sensitive over resistant bacteria.

Furthermore, we also note the Eagle effect in the resistant genotypes. The rugged nature of the fitness landscapes for the R+ and RC strains demonstrates that the *rpsL* K42N resistance mutation does not render these genotypes insensitive to the presence of streptomycin. It is conceivable that a different resistance mechanism such as an efflux pump would simply lower the intracellular drug concentration below the killing threshold while leaving the cell susceptible to other effects an antibiotic may induce. Our results indicate that even resistance mediated by a mutational target does not cause a strain to be completely immune to antibiotics. Furthermore, the acquisition of different compensatory mutations by the resistant strain introduces additional epistatic effects on fitness across drug concentration.

Considering the effects of compensatory mutations on a resistant strain, the question of when and where a compensatory mutation exerts its influence has been addressed in numerous studies with a variety of results. These include broad effects in which a second-site mutation selected under particular experimental conditions is only beneficial in that condition, or is also beneficial in another environmental context, or even the extreme opposite in which a compensatory mutation leads to the dependence of the bacteria to the drug for growth [22–24]. Here, we find that the fitness increase imparted by C mutations selected in the absence of drug continues even when drug is present – that is, RC strains have higher fitness than R+ across the majority of concentrations studied. Where and when the benefits of a compensatory mutation may manifest will affect the trajectory of the resistant genotypes who bear them as they compete in the absence of antibiotics against wild type bacteria, or in the presence of drug treatment where other bacteria may contain resistance determinants that have little or no fitness cost.

One of the most striking findings of this study is our description of second-site mutations that are advantageous independent of the resistance mutation. The +C mutations that have beneficial effects without the resistance mutation, by definition, are not compensatory. However, their absence during exper-

imental evolution of sensitive control lines and the significant proportion in which they are observed among mutations selected in resistant lines is puzzling. There are at least two possible explanations for why these beneficial mutations are not found more frequently.

First, our results consistently show that the fitness of both resistant and sensitive bacteria is dependent on antibiotic concentration, and their fitness can either increase or decrease in response to small increases of dosage. It is therefore likely that many other environmental cues will have strong effects on fitness – the natural and even laboratory environment could then modulate or even determine whether the effects of these mutations will manifest. More specifically, compensatory mutations that may be acquired to alleviate costly resistance in one environment may have a different effect in a resistant or sensitive strain background in a new environment. This may help explain the discrepancy between the frequencies of compensatory mutations observed in laboratory versus clinical studies. While laboratory experiments are most often conducted under similar if not identical conditions, pathogenic bacteria are exposed to drastically different environments as they travel across the body's compartments.

Second, obtaining the resistance mutation could serve as a necessary step towards the acquisition of compensatory mutations that are either deleterious or beneficial in the absence of drug. Previous work has noted a comparable low fitness mutational step as a required intermediate to the acquisition of beneficial mutations. Fong and Palsson found that *E. coli* in whom metabolic genes have been knocked out evolved to have higher fitness than their ancestor [25]. The beneficial ribosomal mutations we characterized could imply that the evolution of costly antibiotic resistance may lead to the acquisition of a novel set of mutations unavailable to the wild type.

**Compensatory mutations and varying antibiotic concentrations increase the number of evolutionary paths available to antibiotic resistant mutants**

As previous theoretical investigations on the influence of compensatory mutations in maintaining costly antibiotic resistance relied on data from only sensitive, resistant, and resistant and compensated strains, and only in a few antibiotic concentrations (namely in the absence or presence of antibiotics), we used our estimates of growth and death rates to re-evaluate this issue. Our model results illustrate how the combination of specific compensatory mutations in different antibiotic-containing environments can produce a wide variety of effects on the population dynamics of sensitive and resistant bacteria.

The result we find most substantial is that in the clinically relevant pharmacodynamic condition, all the 17 scenarios contain both resistant and sensitive bacteria at the end of the observation period. This diversity is facilitated by dependence of the fitness of all genotypes on drug concentration, suggesting a

previously unrecognized role of antibiotic concentration in aiding the reemergence of sensitive bacteria.

As a future direction of this work, we are adapting our model to allow the acquisition of all possible C mutations. Removing the restriction will allow for the competition between different +Cs and RCs and not just between the sensitive and resistant versions of a specific C allele.

Taken together, the combination of our empirical and theoretical investigations on compensatory mutations highlight the importance of both specific alleles and environmental context. Our work serves as the most comprehensive study to date of the effects of mutations acquired in the absence of antibiotics to alleviate the cost of a resistance mutation also in the absence of antibiotics. The drug-dependent fitness landscapes we explored suggest that antibiotic concentration could play an important role in maintaining or disfavoring costly resistance. Our investigation of the relationship between genotype and drug concentration serves as an important initial step in predicting and possibly manipulating the evolutionary trajectory of antibiotic resistant bacteria.

# Methods

### Strains and Media

The variants of the *Salmonella enterica* serovar *Typhimurium* LT2 strain used in this study were originally created and described in Maisnier-Patin et al, Mol Micro 2002 [9]. In brief, the authors asked where and what sort of mutations could compensate for a single costly antibiotic resistant. They replicate and serially passage a streptomycin-resistant strain bearing a K42N mutation in *rpsL* in drug-free broth until they detect an increase in fitness. Target genes from the resulting replicate cultures are then sequenced leading to the identification of compensatory mutations. These single nucleotide substitutions were then separated and transduced into the ancestral drug resistant background, as well as to the ancestral background lacking the resistance mutation. Our study population consists of the ancestral antibiotic sensitive and resistant strains, 17 strains bearing the resistance mutation and a single identified compensatory mutation each, and 17 strains containing only the compensatory mutations (Supplementary Table 1). All experiments are carried out in LB media at 37°C.

### Measurements of Fitness: Growth curves and Kill curves

Fitness as exponential growth rate was assessed for all strains in different streptomycin-containing environments. This was determined as changes in optical density over time at 600 nm ($OD_{600nm}$).

Aside from growth rates measured in sub-inhibitory concentrations of streptomycin, the death rates of all drug sensitive strains were measured by taking cells in exponential phase and adding them to LB broth containing the MIC and 5X the MIC of streptomycin (20ug/mL and 100ug/mL). The resulting culture was then sampled at regular intervals via plating to determine the number of colony-forming units. In addition, some genotypes exhibit increased sensitivity to the drug and the death rates for these strains were also obtained at every streptomycin concentration below 20 ug/mL.

The growth or death rates of all strains were measured in the following concentrations of streptomycin in ug/mL: 0, 0.5, 2.5, 5, 10, 15, 20, 100. Additionally, the growth rates of all resistant strains were measured in the following concentrations: 40, 60, 80, 100, 1000, 10000.

**Model Description**

In order to gain a better understanding of the evolutionary dynamics of compensatory mutations in the presence of drugs at varying concentrations, we constructed a mathematical model of a bacterial population evolving under mutation and natural selection. In this model, we consider the four genotypes ++, R+, C+ and RC, where C can be any of our experimentally studied compensatory mutations. Let $N_i$ be the number of bacteria of genotype i ($i \in$ {++, R+, +C, RC}). The in vitro net growth rate of genotype i at a given drug concentration A is given by our fitted fitness function for that genotype, $w_i(A)$. We assume that this net growth is a composite of (1) baseline growth that is dependent on genotype, (2) natural death at a rate d that is independent of genotype, and (3) additional death imposed by the antibiotics, depending on both genotype and antibiotic concentration. Moreover, we assume that growth is density dependent with carrying capacity K and that under in vivo conditions, growth may reduced by a factor r. Mutations occur at rates $\mu_R$ and $\mu_C$ at the resistance and compensation locus, respectively, and we assume that forward and backward mutations occur at the same rate.

With these assumptions, we arrive at the following system of ordinary differential equations:

$$\frac{dN_{++}}{dt} = N_{++}(rw_{++}^{max} + d)\left(1 - \frac{N}{K}\right) - N_{++}(w_{++}^{max} - w_{++}(A) + d)$$

$$-(\mu_R + \mu_C)N_{++} + \mu_R N_{R+} + \mu_C N_{+C}$$

$$\frac{dN_{R+}}{dt} = N_{R+}(rw_{R+}^{max} + d)\left(1 - \frac{N}{K}\right) - N_{R+}(w_{R+}^{max} - w_{R+}(A) + d)$$

$$-(\mu_R + \mu_C)N_{R+} + \mu_R N_{++} + \mu_C N_{RC}$$

$$\frac{dN_{+C}}{dt} = N_{+C}(rw_{+C}^{max} + d)\left(1 - \frac{N}{K}\right) - N_{+C}(w_{+C}^{max} - w_{+C}(A) + d)$$

$$-(\mu_R + \mu_C)N_{+C} + \mu_R N_{RC} + \mu_C N_{++}$$

$$\frac{dN_{RC}}{dt} = N_{RC}(rw_{RC}^{max} + d)\left(1 - \frac{N}{K}\right) - N_{RC}(w_{RC}^{max} - w_{RC}(A) + d)$$

$$-(\mu_R + \mu_C)N_{RC} + \mu_R N_{+C} + \mu_C N_{R+}$$

Here, $w_i^{max} = w_i(0)$ is the estimated net growth rate of genotype i in the absence of drugs, and $N = N_{++} + N_{R+} + N_{+C} + N_{RC}$ is the total population size. We solved these differential equations numerically using the package deSolve implemented in R (The R Foundation for Statistical Computing; http://www.R-project.org).

For the simulation of pharmacokinetics, we assume that the maximal concentration is reached instantaneously and that the drug concentration decays with a first order kinetics $e^{-kt}$ with k = 0.1.

**Table 1. Parameters used in the model**

| Parameter | Value | Reference |
|---|---|---|
| Carrying capacity (K) | $10^9$ | |
| Mutation rate at resistance locus | $10^{-9}$ | [26] |
| Mutation rate at compensation locus | $10^{-9}$ | [26] |
| Death rate | 1/24 [/h] | [27] |
| Growth rate | 0.092 [/h] | [27] |

## Author contributions

All authors conceived and designed the experiments. PSO performed all the experiments. PSO, JE, and PAzW analyzed the results. JE and PAzW formulated the model. PSO wrote the paper. All authors discussed the results and implications and commented on the manuscript.

# References


1. Fridkin S, Hageman J, Morrison M, Thomson Sanza L, Como-Sabetti K, et al. (2005) Methicillin-resistant Staphylococcus aureus disease in three communities. N Engl J Med 352: 1436–1444.

2. Klein E, Smith DL, Laxminarayan R (2009) Community-associated methicillin-resistant Staphylococcus aureus in outpatients, United States, 1999-2006. Emerg Infect Dis 15: 1925–1930. doi:10.3201/eid1512.081341.

3. Chambers HF, DeLeo FR (2009) Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Micro 7: 629–641.

4. Gustafsson I, Cars O, Andersson DI (2003) Fitness of antibiotic resistant Staphylococcus epidermidis assessed by competition on the skin of human volunteers. J Antimicrob Chemother: 258–263. doi:10.1093/jac/dkg331.

5. Andersson DI, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2: 489–493.

6. Schrag SJ, Perrot V, Levin BR (1997) Adaptation to the fitness costs of antibiotic resistance in Escherichia coli. Proc Roy Soc B 264: 1287–1291. doi:10.1098/rspb.1997.0178.

7. Reynolds MG (2000) Compensatory Evolution in Rifampin-Resistant. Genetics 156:1471-81.

8. Maisnier-Patin S, Andersson DI (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol 155: 360–369. doi:10.1016/j.resmic.2004.01.019.

9. Maisnier-Patin S, Berg OG, Liljas L, Andersson DI (2002) Compensatory adaptation to the deleterious effect of antibiotic resistance in. Mol Microbiol: 355–366.

10. Cortes PR, Cian MB, Albarracı AG (2011) Compensatory Evolution of pbp Mutations Restores the Fitness Cost Imposed by b-Lactam Resistance in Streptococcus pneumoniae. PLoS Pathog 7: e1002000. doi:10.1371/journal.ppat.1002000.

11. Nagaev I, Björkman J, Andersson DI, Hughes D (2001) Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. Mol Microbiol 40: 433–439.

12. Böttger E, Springer B, Pletschette M, Sander P (1998) Fitness of antibiotic-resistant microorganisms and compensatory mutations. Nature Medicine 4: 11–12.

13. Paulander W, Maisnier-Patin S, Andersson DI (2007) Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in Salmonella typhimurium. Mol Microbiol 64: 1038–1048. doi:10.1111/j.1365-2958.2007.05713.x.

14. Meka VG, Gold HS, Cooke A, Venkataraman L, Eliopoulos GM, et al. (2004) Reversion to susceptibility in a linezolid-resistant clinical isolate of Staphylococcus aureus. J Antimicrob Chemother 54: 818–820.

15. Luo N, Pereira S, Sahin O, Lin J, Huang S, et al. (2005) Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. Proc Natl Acad Sci USA 102: 541–546. doi:10.1073/pnas.0408966102.

16. Huang T-D, Almpanis C, Denis O, Nonhoff C, Delaere B, et al. (2007) Reversion of resistance in relapsing infection caused by a glycopeptide-intermediate methicillin-resistant Staphylococcus aureus isolate. Eur J Clin Microbiol Infect Dis 26: 419–422. doi:10.1007/s10096-007-0297-1.

17. Schulz zur Wiesch P, Engelstädter J, Bonhoeffer S (2010) Compensation of fitness costs and reversibility of antibiotic resistance mutations. Antimicrob Agents Chemother 54: 2085–2095. doi:10.1128/AAC.01460-09.

18. Tanaka M, Valckenborgh F (2011) Escaping an Evolutionary Lobster Trap: Drug Resistance and Compensatory Mutation in a Fluctuating Environment. Evolution 65: 1376–1387. doi:10.1111/j.1558-5646.2011.01223.x.

19. Bergstrom C, Feldgarden M (2008) The ecology and evolution of antibiotic-resistant bacteria. In: Stearns S, Koella J, editors. Evolution in health and disease. New York: Oxford Univ Press. pp. 125–137.

20. Zhu M, D P, Burman WJ, Jaresko GS, D P, et al. (2001) Population Pharmacokinetics of Intravenous and Intramuscular Streptomycin in Patients with Tuberculosis. Pharmacother 21(9):1037-45 .

21. Eagle H, Musselman A (1948) The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J Exp Med 88(1): 99-131.

22. Baptista M, Depardieu F, Reynolds P, Courvalin P, Arthur M (1997) Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. Mol Microbiol 25: 93–105.

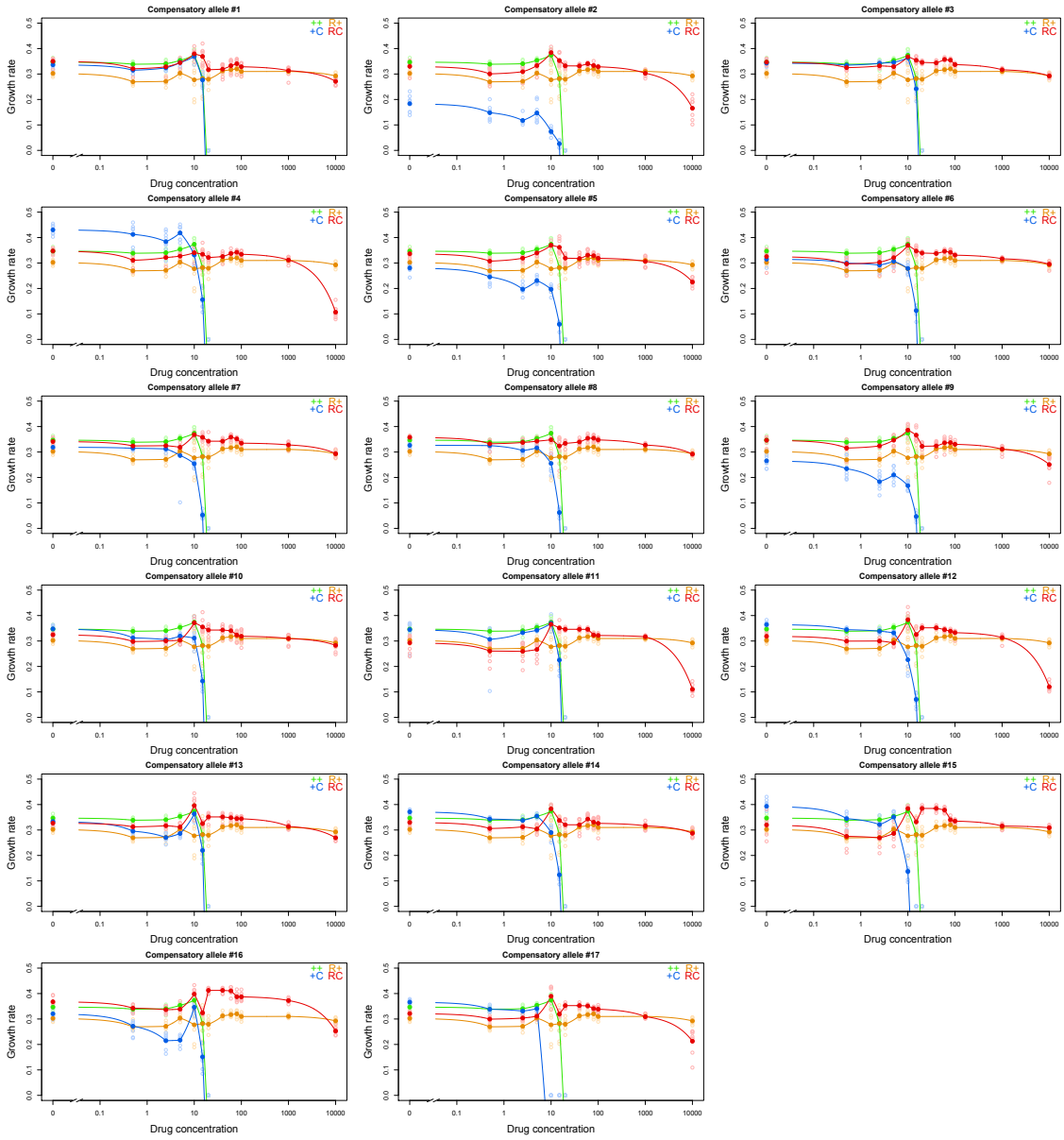
23. Périchon B, Courvalin P (2009) VanA-type vancomycin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 53: 4580–4587. doi:10.1128/AAC.00346-09.

24. Moubareck C, Meziane-Cherif D, Courvalin P, Périchon B (2009) VanA-type Staphylococcus aureus strain VRSA-7 is partially dependent on vancomycin for growth. Antimicrob Agents Chemother 53: 3657–3663. doi:10.1128/AAC.00338-09.

25. Fong SS, Palsson BØ (2004) Metabolic gene-deletion strains of Escherichia coli evolve to computationally predicted growth phenotypes. Nature Genetics 36: 1056–1058. doi:10.1038/ng1432.

26. Drake JW (1991) A constant rate of spontaneous mutation in DNA-based microbes. Proc Natl Acad Sci USA 88: 7160–7164.

27. Grant AJ, Restif O, Mckinley TJ, Sheppard M, Maskell DJ, et al. (2008) Modelling within-Host Spatiotemporal Dynamics of Invasive Bacterial Disease. PLoS Biol 6: e74. doi:10.1371/journal.pbio.0060074. doi:10.1371/journal.pbio.0060074.




**Supplementary Figure 1. Fitness landscapes of all strains used in the study.**

**Supplementary Table 1. List of strains and genotypes used in the study**

| Symbol | Genotype | C Mutation |
|---|---|---|
| ++ | ancestral sensitive | |
| R+ | *rpsL* K42N | |
| 1 | *rpsL* K42N | *rpsD* D49Y |
| 2 | | *rpsD* D49Y |
| 3 | *rpsL* K42N | *rpsD* Q53L |
| 4 | | *rpsD* Q53L |
| 5 | *rpsL* K42N | *rpsD* N84D |
| 6 | | *rpsD* N84D |
| 7 | *rpsL* K42N | *rpsD* N84S |
| 8 | | *rpsD* N84S |
| 9 | *rpsL* K42N | *rpsD* T85I |
| 10 | | *rpsD* T85I |
| 11 | *rpsL* K42N | *rpsD* E201G |
| 12 | | *rpsD* E201G |
| 13 | *rpsL* K42N | *rpsD* E201V |
| 14 | | *rpsD* E201V |
| 15 | *rpsL* K42N | *rpsD* S204F |
| 16 | | *rpsD* S204F |
| 17 | *rpsL* K42N | *rpsD* S204T |
| 18 | | *rpsD* S204T |
| 19 | *rpsL* K42N | *rpsE* A98V |
| 20 | | *rpsE* A98V |
| 21 | *rpsL* K42N | *rpsE* G101A |
| 22 | | *rpsE* G101A |
| 23 | *rpsL* K42N | *rpsE* I105S |
| 24 | | *rpsE* I105S |
| 25 | *rpsL* K42N | *rpsE* G108A |
| 26 | | *rpsE* G108A |
| 27 | *rpsL* K42N | *rpsE* V122A |
| 28 | | *rpsE* V122A |
| 29 | *rpsL* K42N | *rpsE* T130I |
| 30 | | *rpsE* T130I |
| 31 | *rpsL* K42N | *rplS* Q40R |
| 32 | | *rplS* Q40R |
| 33 | *rpsL* K42N | *rplS* Q40L |
| 34 | | *rplS* Q40L |

# 3

# Modified growth of streptomycin-induced streptomycin resistant bacteria.

**Paolo S. Ocampo[1,3], Martin Ackermann[2,3], Sebastian Bonhoeffer[1]**

[1]Institute of Integrative Biology, ETH Zurich
[2]Institute of Biogeochemistry and Pollution Dynamics, ETH Zurich
[3]Department of Environmental Microbiology, EAWAG

# Abstract

Aside from death, antibiotics induce many different morphologic, physiologic, and genetic changes in drug sensitive bacteria. It is currently not clear, however, whether antibiotics can similarly affect antibiotic resistant bacteria, or what kind of changes this may include. In studying the costs of resistance to one drug in antibiotic stress, we found an unexpected effect of streptomycin on the fitness of future generations of streptomycin-resistant (SmpR) bacteria in a second drug. Although sensitive and SmpR *E. coli* first grown in plain media for 24 hours both displayed slightly decreased growth rates when transferred to fresh media supplemented with sub-inhibitory chloramphenicol, SmpR bacteria first grown in streptomycin and then exposed to low chloramphenicol levels exhibited growth rates significantly higher than wild type bacteria in the absence of any drug. This priming effect of streptomycin on SmpR strains was found to be transient, specific for chloramphenicol, and required a concentration of streptomycin that is inhibitory to sensitive cells. Whole genome sequencing needs to be performed to determine the mutations underlying this mechanism. In this chapter, we describe the experiments performed to define this novel phenomenon, and discuss the implications this may have on our understanding of antibiotic resistance.

# Introduction

The costs associated with a resistance determinant are among the most important factors in determining both the speed and extent of the emergence of resistance [1–4]. Bacteria have at their disposal a variety of mechanisms by which they reduce or even remove these costs. These include the evolution of a second-site compensatory mutation (see Chapter 2), or by evolving regulatory mechanisms that control the expression of resistance genes [5].

An important consideration in measuring these costs is that environmental conditions have been found to have significant influence. As an example, some specific resistance mutations that have been shown to be cost-free in *in vitro* experiments have high costs when grown in laboratory mice, and the reverse has also been reported with costs revealed in laboratory media and absent in mice [6,7].

Much more generally, Kishony and Leibler have shown that a variety of environmental stressors can reduce the average cost of random deleterious mutations [8]. Among these cost-alleviating stressors they identified are sub-inhibitory concentrations of two antibiotics of differing mechanisms of action, chloramphenicol and trimethoprim.

In this study, we initially sought to determine whether these same stressors – low concentrations of antibiotics - were able to reduce the cost of defined deleterious mutations, specifically antibiotic resistance mutations. Recent studies suggest that antibiotics used in human or veterinary medicine may be leaking into the environment and influencing the rise of resistant infections [9]. Determining whether sub-inhibitory concentrations of various antibiotics alleviate the burden of costly resistance could aid in defining the link between antibiotic pollution and community-acquired resistant infections.

Unforeseen results lead to a reorientation of the project to instead characterize a novel phenomenon. The original experimental design involved obtaining resistant bacteria. This was achieved by plating an overnight culture of *E. coli* on solid LB media supplemented with streptomycin to select for streptomycin resistant (SmpR) mutants. The growth rates of the resulting strains would be measured in the presence of antibiotic stress. In contrast to previous work on random deleterious mutations, we did not find that exposure to sub-inhibitory concentrations of a second antibiotic alleviated the cost of resistance. Instead, we found that the growth conditions 24 hours *prior* to the experimental treatment had a strong effect on fitness. In this chapter, I report on the experiments performed to describe this novel, environment-specific effect and discuss the possible implications this may have on the current concept of antibiotic resistance.

# Results

These results are based on a set of 20 SmpR *E. coli* strains that were generated via plating on LB agar supplemented with 100 ug/mL of streptomycin over 48 hours. The general design of the experiment involves streaking a frozen culture of the previously isolated SmpR strains – or wild-type strains as controls - onto a plain LB plate followed by incubation overnight at 37°C. Eight individual colonies of each strain are used to each inoculate a single well in a 96-well plate containing media with or without streptomycin (pre-culture performed on day 0). After overnight incubation, the pre-culture is diluted 10,000x into a second 96-well plate containing fresh media with or without a second antibiotic (experimental condition performed on day 1).

Resistant strains pre-cultured in plain media and then exposed to sub-inhibitory concentrations of chloramphenicol (0.2 ug/mL) had slightly lower fitness than wild type. However, pre-culture in streptomycin primed the resistant strains to display significantly increased growth in chloramphenicol (Fig. 1). Strikingly, primed resistant strains in chloramphenicol have significantly higher growth than the ancestral strain in plain media.

It is important to note that repeated attempts at isolating SmpR strains that display this effect by plating new cultures of the ancestral strain on streptomycin have been unsuccessful. The growth rate measurements in chloramphenicol for the available strains, however, have been replicated many times and results have been consistent.

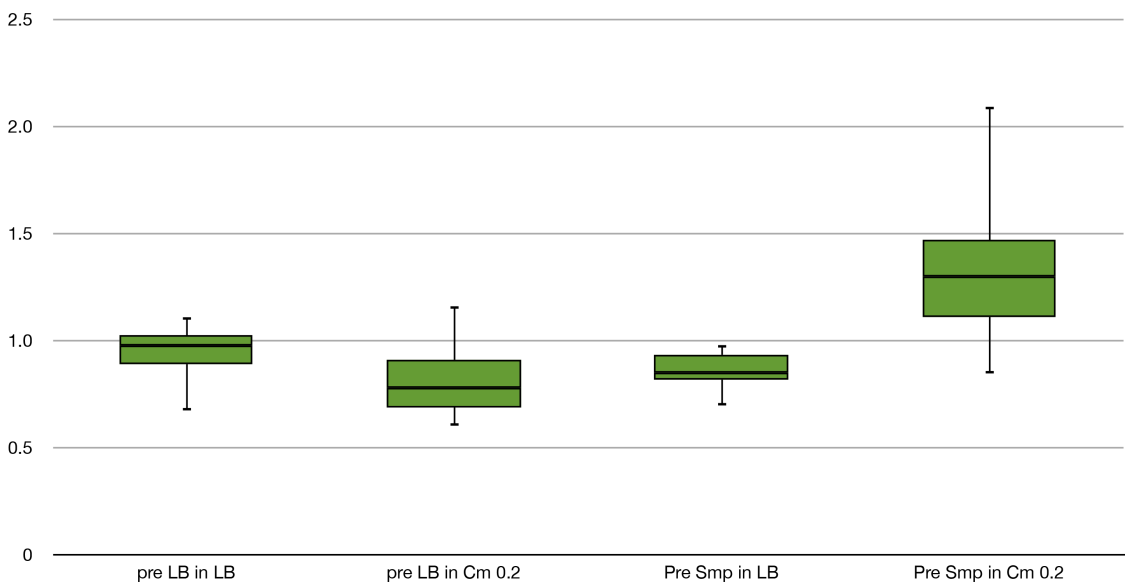



**Figure 1. High concentrations of streptomycin increase transgenerational fitness in sub-inhibitory chloramphenicol.**

Each boxplot depicts the fitness of 20 SmpR strains in four experimental treatments relative to the ancestral wild type measured in plain LB. The conditions from L to R are as follows: (1) pre-cultured in LB and then measured in LB, (2) pre-cultured in LB and then measured in chloramphenicol, (3) pre-cultured in streptomycin and then measured in LB, and (4) pre-cultured in streptomycin and then measured in chloramphenicol. While cells in condition (2) had significantly lower fitness than (1), unpaired, two-tailed t-test assuming unequal variance, $p = 0.0003$, changing the pre-culture treatment to condition (4) resulted in significantly higher fitness, $p<10^{-12}$. The edges of the box mark the $25^{th}$ and $75^{th}$ percentile, the horizontal line marks the median, the whiskers denote the maximum and minimum of all the data.

**Priming is antibiotic-specific**

The experimental treatment was repeated with the substitution of a sub-inhibitory concentration of the folic acid synthesis inhibitor trimethoprim (0.2 ug/mL) instead of chloramphenicol (Figure 2). The fitness of the resistant strains pre-cultured in either plain broth or with streptomycin was both found to decrease slightly and were not significantly different from each other. Growth rate measurements performed in two other concentrations of trimethoprim, 0.02 and 0.1 ug/mL, did not produce any discernible effect on fitness of the resistant strains (results not shown).

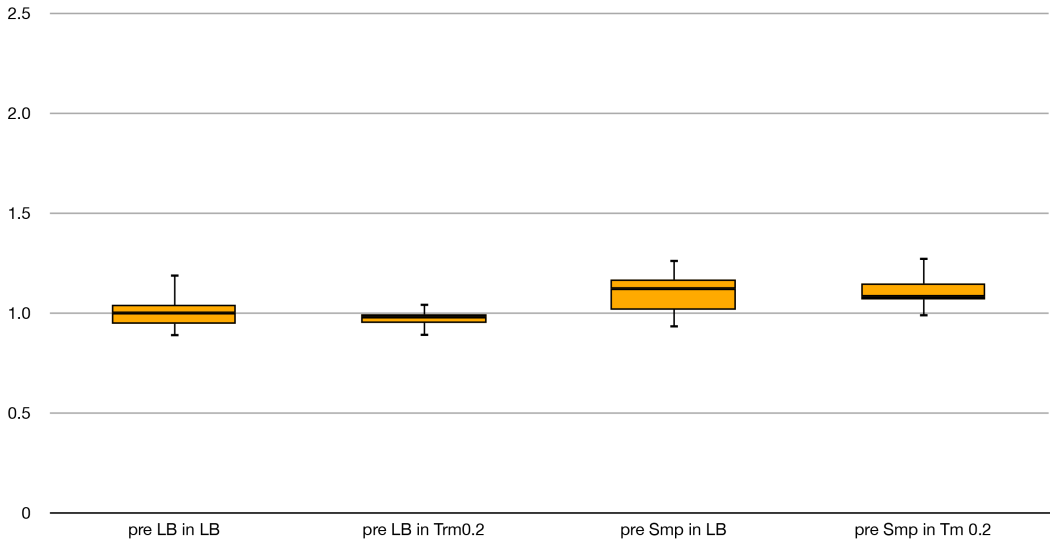



**Figure 2. Transgenerational fitness benefit is not induced by sub-inhibitory concentrations of trimethoprim.**

The relative fitness of SmpR strains is not significantly different from the wild type during exposure to the folate inhibitor trimethoprim. (unpaired, two-tailed t-test assuming unequal variance, p=0.77)

**Increased fitness is partly due to an interaction of chloramphenicol and dilute concentrations of streptomycin**

Since there was no washing step in between the pre-culture and experimental conditions, it is possible that the high fitness observed by primed strains in a second antibiotic is due to a direct interaction between diluted streptomycin and chloramphenicol. To test this, un-primed strains were grown in sub-inhibitory chloramphenicol and a range of low streptomycin concentrations. To rule out whether the incubation step had an effect on streptomycin, LB with 100 ug/mL streptomycin was shaken at 37°C without cells and then diluted 10,000x before being applied to resistant cultures with chloramphenicol (Figure 3). The combination of a low streptomycin concentration and chloramphenicol induced a fitness increase significantly higher than strains pre-cultured in LB and grown in LB (unpaired, two-tailed t-test assuming unequal variance, p=0.02). However, the magnitude of the increase of growth rate of strains in the combination treatment is significantly lower than the magnitude of the increase observed in primed strains (unpaired, two-tailed t-test assuming unequal variance, p=0.0002). This indicates that the combination of low streptomycin and low chloramphenicol is at least partially responsible for the fitness increase we observe, but does not explain its full magnitude, suggesting that priming has a significant effect on its own.

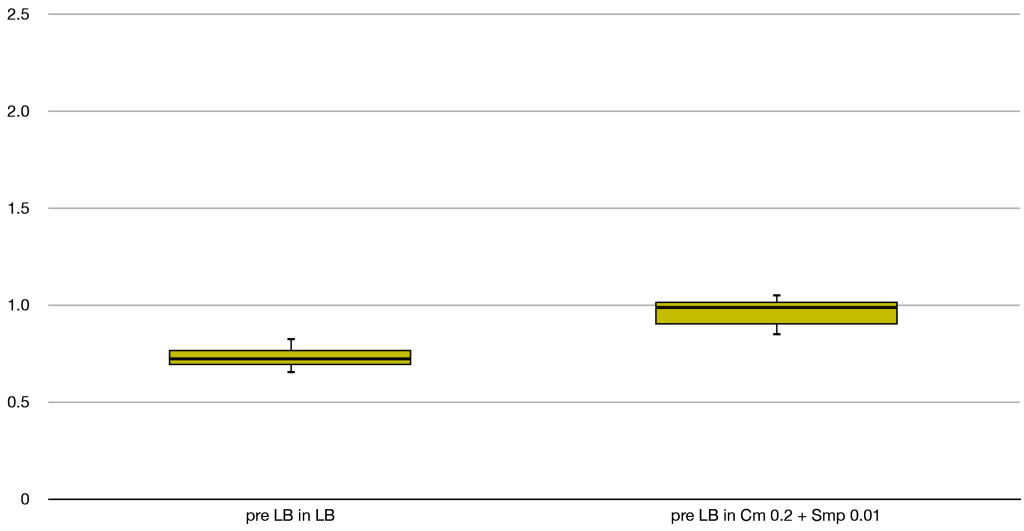



**Figure 3. Transgenerational fitness benefit is partially the product of sub-inhibitory chloramphenicol and diluted streptomycin.**

The fitness difference between the population of resistant strains pre-cultured in LB and then grown either in LB or the combination of 0.2 ug/mL chloramphenicol and 0.01 ug/mL streptomycin is significant (unpaired, two-tailed t-test assuming unequal variance, p = 0.02). The significant difference in the magnitude of the increase in growth rate between strains exposed to the combination condition and strains pre-cultured in streptomycin before growth in chloramphenicol suggests that the combination condition is only partially responsible (unpaired, two-tailed t-test assuming unequal variance, p=0.0002).

## Priming effect is streptomycin-concentration dependent

The standard experimental condition to induce the increased growth effect in chloramphenicol was to prime the SmpR strains in the concentration of streptomycin used to initially select for resistance (100 ug/mL). Lowering the streptomycin concentration used in priming to 10 ug/mL or 1 ug/mL resulted in the absence of increased growth in chloramphenicol (Figure 4).

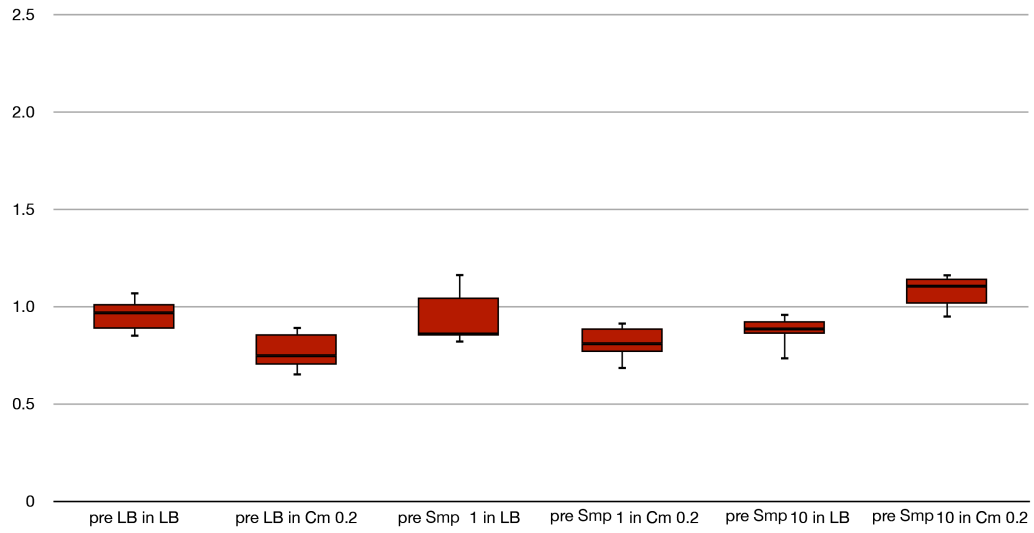



**Figure 4. Priming with sub-inhibitory concentrations of streptomycin does not produce the transgenerational fitness benefit during exposure to sub-inhibitory chloramphenicol.**

Cells pre-cultured in LB and grown in Cm 0.2 exhibited lower growth rates than cells pre-cultured in LB and grown in LB (unpaired, two-tailed t-test assuming unequal variance, $p = 0.0003$). Considering the effect of sub-inhibitory streptomycin in the pre-culture condition, exposure to 1 ug/mL of streptomycin resulted in a significant decrease in growth rate in Cm 0.2 compared to LB (unpaired, two-tailed t-test assuming unequal variance, $p = 0.03$). However pre-culture in 10 ug/mL of streptomycin resulted in a significant increase in growth in Cm 0.2 compared to cells pre-cultured in LB and grown in LB (unpaired, two-tailed t-test assuming unequal variance, $p = 0.05$ respectively).

### Effect is transient and not stably inherited

In order to determine whether the effect in chloramphenicol induced by streptomycin was permanent or transient, the experimental design was modified to include a passage in fresh LB broth for 24 hours at 37°C between the pre-culture condition and the experimental condition. This resulted in strains from either pre-culture condition displaying growth rates that were not significantly different (Figure 5).

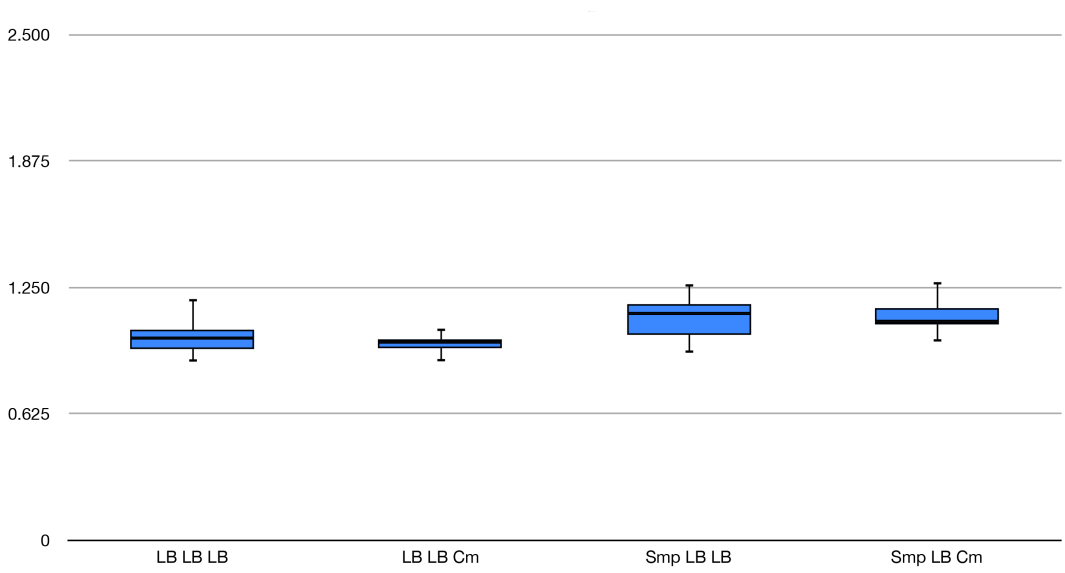



**Figure 5. The addition of a serial passage step resulted in the negation of the transgenerational fitness benefit in sub-inhibitory chloramphenicol.**

Pre-culturing in Smp, followed by passaging in LB, and then growth in Cm did not vary significantly from pre-culturing in Smp, passaging in LB, and growth in LB (unpaired, two-tailed t-test assuming unequal variance, $p = 0.85$)

### Priming with streptomycin does not result in resistance to chloramphenicol

Each of the 20 SmpR strains were grown in LB with or without streptomycin at 37°C overnight. The resulting culture was used to perform a standard minimum inhibitory concentration (MIC) estimate for chloramphenicol. All cultures, regardless of pre-culture conditions, had an MIC for chloramphenicol of 5 ug/mL.

### Streptomycin resistance mutations

Sequencing was performed on the *rpsL* gene of all recovered SmpR clones. Results reveal 4 different well-characterized streptomycin resistance mutations. Of the 20 different clones, eight bear the K43N mutation, six have K88R, four contain K42T, and two have K87R. A one-way analysis of variance yielded no

significant differences between the transgenerational benefit of the four different mutations, $F(3,13) = 0.667$, $p = 0.58$.

# Discussion

Dependent on concentration, we are aware of a wide variety of responses induced by antibiotics – in sensitive bacteria. In contrast, little consideration is paid to how antibiotics at any concentration affect resistant bacteria. Here, I add to the understanding of how antibiotics influence resistant bacteria by describing a novel phenomenon in which the succeeding generations of resistant bacteria may experience additional benefits. Specifically, the experiments performed in this chapter draw attention to three principal findings:

First, that the acquisition of streptomycin resistance may lead to effects unrelated to survival in the presence of a drug. It must first be noted that although bacteria that have acquired resistance mutations will obviously thrive in high levels of a particular drug, they may not be completely insensitive to the presence of that drug. In Chapter 2, I show how the dose-response curves for many different resistant genotypes in a wide range of antibiotic concentrations display non-monotonic changes in growth rate as antibiotic concentration increases. Conceivably, this could occur via the formation of weak, highly reversible bonds between streptomycin and its binding site on the bacterial ribosome. This could result in perturbations to normal cell physiology that manifests as fluctuations in growth rate. The results I present in this chapter suggest that the acquisition of streptomycin resistance mutations may produce additional effects besides increased survival in streptomycin. Three examples of such unforeseen effects are discussed below; these include increased ribosomal accuracy, differences in virulence, and increased growth in the presence of a second antibiotic.

Mutations that grant streptomycin resistance can be divided into two classes: Error-restrictive mutations on *rpsL* increase the accuracy of inspection of codon-anticodon pairings on the ribosomal A site, resulting in decreased rates of peptide elongation and decreased growth rates [10]. Non-restrictive mutations have also been described and these have similar accuracy and growth rate as wild type strains. Interestingly, these classes are allele-specific – for example, K42T and K42N are both restrictive while K42R is not [11].

Furthermore, the acquisition of different types of resistance mutations has been found to result in disparities in other traits such as virulence. The foodborne pathogen, enterohemorrhagic *E. coli* O157:H7 containing restrictive streptomycin resistance mutations were found to have decreased expression and secretion of EspA and EspB, two proteins required for attaching and effacing to

the intestinal epithelium. In contrast, nonrestrictive mutants displayed little or insignificant changes in secretion levels [12].

Interestingly, a phenomenon related to the fitness benefit described in this chapter has been recently reported. Pelchovich and colleagues asked whether the structural alterations that accompany streptomycin resistance mutations would affect sensitivity to other antibiotics that target the ribosome [13]. Although all their pre-culture conditions involved growth in LB broth, they observed a range of fitness effects during exposure to unrelated antibiotics. Decreased growth rates occurred in SmpR strains grown in sub-lethal chloramphenicol and tetracycline; in contrast they also found that these mutants had higher growth rates than wild type bacteria in a sub-inhibitory concentration of gentamicin. These results provide additional support for a strain bearing a resistance mutation to experience higher fitness in the presence of an antibiotic to which it is sensitive. This may suggest a weak form of cross-resistance. The results detailed in this chapter, however, indicate that investigations on the effects of antibiotics on the fitness of resistant bacteria should also consider the preculture condition.

Second, the phenomenon I describe is broadly consistent with other cryptic or unrecognized bacterial mechanisms in terms of reliance on concentration. Since antibiotics specifically target essential components of bacterial physiology, it is conceivable that the degree of an effect or even different kinds of effects can accompany increasing drug concentrations. For example, sub-inhibitory concentrations of the fluoroquinolone ciprofloxacin has been found to potentiate the production of cytolethal distending toxin, an important component in the pathogenesis of *C. jejuni* [14]. At higher concentrations, fluoroquinolones induce the SOS response and this promotes many responses such as the formation of persisters [15]. Awareness of these effects could aid in devising dosage strategies that avoid the induction of concentration-dependent responses, or capitalize on them in a way that maximizes treatment efficacy.

The last important consideration raised by these experiments is the transgenerational but transient effects antibiotics may produce in resistant strains. In a related fashion, transient responses of bacteria to antibiotics is explored in Chapter 4 (See Figure 3 for filamentation during exposure to some drugs) and discussed further in Chapter 5. Conceptually, this transgenerational effect could be linked to the well-studied general stress response of bacteria. The SOS response is triggered by a wide range of environmental cues, including many antibiotics, and could aid the future success of bacterial progeny. These could be transient effects such as persistence, or permanent changes including the evolution of resistance in daughter cells via error-prone DNA replication or recombination of divergent sequences, or the acquisition of mobile genetic elements

that often harbor resistance genes [15,17,18]. The effect we observe in SmpR *E. coli* appears heritable to a certain extent and then ceases, and is contingent on an unexpected environmental context – the presence of inhibitory concentrations of streptomycin.

**Future direction**

Whole genome sequencing is currently being performed in order to determine whether this effect has a detectable genetic basis. Aside from this, a number of experiments are planned to better understand this phenomenon:

First, observations at the single cell level using microfluidic devices are to be performed on these mutants to determine whether all or only a few individuals are responsible for the increased growth rates observed after pre-culture in streptomycin. We envision at least two possible scenarios underlying the increased growth of pre-treated SmpR strains in chloramphenicol that we hope to clarify using these experiments.

It is possible that the pre-culture condition involving a sufficiently high streptomycin concentration may enrich the population by selecting for certain genotypes or phenotypes. This sub-population of cells may exhibit increased growth while the rest does not. Conversely, the pre-culture condition may also induce a physiological change in all cells.

Moreover, determining whether there are differences in growth rates among individual cells may help identify an interaction between cell types that could explain increased growth of these SmpR strains in chloramphenicol. A similar phenomenon has been reported by Lee and colleagues as they examined the population dynamics of bacteria during drug exposure [16]. They began by following a continuous culture of *Escherichia coli* exposed to increasing drug concentrations and found that most individuals were more sensitive than the population as a whole. They isolated a few highly resistant mutants and determined how these cells improve the survival of the whole population by producing indole in response to the presence of antibiotics. Transcriptional profiling revealed how this signaling molecule induces drug efflux pumps and oxidative-stress protective mechanisms in sensitive members of the population. These observations demonstrate how a few resistant individuals can influence a susceptible population to survive antibiotic treatment without explicitly evolving resistance.

Second, these experiments are to be repeated using additional antibiotics to determine the extent of this effect across different environments. The chloramphenicol-specific nature of the inducible fitness increases we measure highlights the importance of placing antibiotic resistance in an assortment of environmental contexts. Various environments as well as various stressors, including antibiotics, have already been revealed to produce a wide selection of re-

sponses from sensitive bacteria. These include transitioning from normal, bacillary growth to filamentous growth, or even stopping growth entirely (see Chapter 4 and 5 for a more detailed discussion of filamentation and bacteriostasis). It is thought that revealing this "hidden" variation of responses in stressful environments results from perturbations on normal homeostasis; this in turn may itself be adaptive as an increase in variation may lead to the selection of novel beneficial adaptations [19]. Conversely, "hidden" variation may also include adverse affects. This is evident when placing the effects of *fusA* mutations in different environmental contexts. These mutations grant fusidic acid resistance to *S. enterica* by altering expression of the transcriptional regulator guanosive tetraphosphate (ppGpp). This results in pleiotropic effects on gene expression that lead to environment-dependent fitness differences, including hypersensitivity to unrelated antibiotics [20]. Our results suggest that uncovering "hidden" variation in fitness in new environments can be an inducible event. We show that increased growth in stressful environments can be observed in SmpR bacteria if they are first exposed to streptomycin.

Finally, we plan to further investigate the combined effect of low streptomycin and low chloramphenicol in increasing growth rate (detailed in Figure 3). The next chapter in this thesis considers every pairwise combination of 21 different antibiotics and the effect these pairs may have on the growth and death of wild type *E. coli*. The results reported in this chapter suggest that probing how a wide range of different concentration combinations may affect bacterial physiology could lead to the identification of novel phenomena.

Currently, we can only speculate as to what molecular mechanism may underlie this novel phenomenon. Regardless of the mechanism that leads to this increase in growth rate, the significant effect of streptomycin on SmpR bacteria draws attention to how our understanding of antibiotic resistance may be incomplete. These results highlight the importance of drug concentration and environmental context in studying resistance.

# Methods

### Strains and Media

All strains were derived from an *Escherichia coli* MG1655 lacZ::gfp strain obtained from the lab of Marjan van der Woude [21]. 20 streptomycin resistant variants were selected via plating of an overnight culture onto LB plates containing 100 ug/mL streptomycin. Sequencing of the *rpsL* gene of these strains revealed only previously reported streptomycin resistance mutations. All experiments were performed in LB broth at 37°C.

## Growth rate measurements

Fitness as exponential growth rate was determined as changes in optical density over time at 600 nm ($OD_{600nm}$). In brief, the ancestral sensitive strain was grown overnight at 37°C in LB before being diluted 10000x into fresh media with or without drugs. All 20 streptomycin resistant strains were similarly pre-cultured in plain broth, or in broth supplemented with streptomycin, before being subjected to the same growth conditions as the ancestral strain. The relative fitness of each strain was calculated as a ratio of the growth rate of the ancestral wild type strain pre-cultured in LB and measured in LB divided by the growth rate of the mutant strain in the appropriate pre-culture and experimental condition.

## Statistical analysis

All statistical tests were performed in JMP.

## Author contributions

All authors conceived and designed the experiments. PSO performed the experiments and wrote the paper. All authors discussed the results and implications and commented on the manuscript.

# References


1. Böttger E, Springer B, Pletschette M, Sander P (1998) Fitness of antibiotic-resistant microorganisms and compensatory mutations. Nature Medicine 4: 11–12.
2. Maisnier-Patin S, Andersson DI (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol 155: 360–369. doi:10.1016/j.resmic.2004.01.019.
3. Andersson DI (2003) Persistence of antibiotic resistant bacteria, Curr. Opin Microbiol 6: 452–456.
4. Andersson DI (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol 9: 461–465. doi:10.1016/j.mib.2006.07.002.
5. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, et al. (2009) Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect Dis 9: 357–364. doi:10.1016/S1473-3099(09)70105-7.
6. Nagaev I, Björkman J, Andersson DI, Hughes D (2001) Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. Mol Microbiol 40: 433–439.
7. Björkman J, Nagaev I, Berg OG, Hughes D, Andersson DI (2000) Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance. Science 287: 1479–1482. doi:10.1126/science.287.5457.1479.
8. Kishony R, Leibler S (2003) Environmental stresses can alleviate the average deleterious effect of mutations. J Biol: 1–10.
9. Hawkey PM, Jones AM (2009) The changing epidemiology of resistance. J Antimicrob Chemother 64 Suppl 1: i3–10. doi:10.1093/jac/dkp256.
10. Agarwal D, Gregory ST, O'Connor M (2011) Error-prone and error-restrictive mutations affecting ribosomal protein S12. J Mol Biol 410: 1–9. doi:10.1016/j.jmb.2011.04.068.
11. Tubulekas I, Buckingham RH, Hughes D (1991) Mutant ribosomes can generate dominant kirromycin resistance. J Bacteriol 173: 3635–3643.

12. Chen C, Blumentritt C a, Curtis MM, Sperandio V, Torres AG, et al. (2013) Restrictive streptomycin-resistant mutations decrease the formation of attaching and effacing lesions in Escherichia coli O157:H7 strains. Antimicrob Agents Chemother. doi:10.1128/AAC.00709-13.

13. Pelchovich G, Schreiber R, Zhuravlev A, Gophna U (2013) The contribution of common rpsL mutations in Escherichia coli to sensitivity to ribosome targeting antibiotics. Int J Med Microbiol. doi:10.1016/j.ijmm.2013.07.006.

14. Ismaeel AY, Senok AC, Bindayna KM, Bakhiet M, Al Mahmeed A, et al. (2005) Effect of antibiotic sub inhibitory concentration on cytolethal distending toxin production by Campylobacter jejuni. J Infect 51: 144–149. doi:10.1016/j.jinf.2004.09.012.

15. Dörr T, Lewis K, Vulić M (2009) SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet 5.

16. Lee HH, Molla MN, Cantor CR, Collins JJ (2010) Bacterial charity work leads to population-wide resistance. Nature 467: 82–85. doi:10.1038/nature09354.

17. López E, Elez M, Matic I, Blázquez J (2007) Antibiotic-mediated recombination : ciprofloxacin stimulates SOS-independent recombination of divergent sequences in Escherichia coli. Mol Microbiol 64: 83–93. doi:10.1111/j.1365-2958.2007.05642.x.

18. Beaber JW, Hochhut B, Waldor MK (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427: 72–74. doi:10.1038/nature02241.

19. Badyaev A V (2005) Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. Proc Roy Soc B 272: 877–886. doi:10.1098/rspb.2004.3045.

20. Macvanin M, Hughes D (2005) Hyper-susceptibility of a fusidic acid-resistant muant of Salmonella to different classes of antibiotics. FEMS Microbiol Lett 247: 215–220.

21. Goulian M, van der Woude M (2006) A simple system for converting lacZ to gfp reporter fusions in diverse bacteria. Gene 372: 219–226. doi:10.1016/j.gene.2006.01.004.

# 4

# Antagonism is prevalent between bacteriostatic and bactericidal antibiotics

**Paolo S. Ocampo[1,4], Viktória Lázár[2], Balázs Bogos[2], Pia Abel zur Wiesch[3], Markus Arnoldini[4,5], Martin Ackermann[4,5], Csaba Pál[2], Sebastian Bonhoeffer[1]**

[1]Institute of Integrative Biology, ETH Zurich

[2]Synthetic and Systems Biology Unit, Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences

[3]Department of Global Health and Social Medicine, Harvard Medical School

[4]Institute of Biogeochemistry and Pollution Dynamics, ETH Zurich

[5]Department of Environmental Microbiology, EAWAG

# Abstract

Combination therapy is rarely used to counter resistance evolution in bacterial infections. An expansion of combination therapy requires knowledge of how drugs at inhibitory concentrations interact. Such knowledge is essential for exploiting the benefits of combination therapy. Previously it has been suggested that if bactericidal drugs are most potent on actively dividing cells, then the inhibition of growth induced by a bacteriostatic drug should result in a reduction of drug efficacy. Our goal here was to investigate this hypothesis systematically. We performed an initial screen of pairwise combinations across 21 different antibiotics at sub-inhibitory concentrations, and found that combining a bacteriostatic with a bactericidal drug has a strong tendency to result in antagonism. Using a subset of these pairs, we extended the analysis to high drug concentrations by constructing time-kill curves, and verified the antagonistic interactions between bacteriostatic and bactericidal drugs at clinically relevant drug concentrations. As our hypothesis relies on a phenotypic effect produced by different drug classes, we recreated these experiments in a microfluidic device and performed time-lapse analyses to directly observe and quantify growth and division of individual cells under controlled antibiotic concentrations. While our single-cell observations supported the antagonism between bacteriostatic and bactericidal drugs, they revealed an unexpected variety of cellular responses to antagonistic drug combinations, suggesting that multiple mechanisms underlie this interaction. These results provide a necessary step towards exploiting drug interactions for the treatment of bacterial infections and the prevention of resistance evolution.

# Introduction

The problem of antibiotic resistance requires a solution that relies on more than just the continuous development of new drugs. Pathogens have been unrelentingly cunning in evolving mechanisms by which to survive in the face of every drug put on the market. Our response must be careful and far-sighted. Combination therapy, the concurrent application of two or more antibiotics, provides an appealing regimen that demands closer assessment as a tool to combat this problem. In the treatment of important infectious diseases such as HIV, tuberculosis, and malaria, combination therapy has become the gold standard precisely to delay the evolution of drug resistance [1–4]. In contrast, among common acute bacterial infections, combinations of drugs are only prescribed in a very limited number of cases and for a different rationale. For those specific instances, two drugs are prescribed for their synergistic effect, that is, for the fact that their combined effect exceeds the sum of their individual effects. Drug synergy had been demonstrated to affect clearance of the infection more efficiently and at lower drug concentrations [5]. Examples of such cases include fusidic acid and rifampin for the treatment of methicillin-resistant *S. aureus* or trimethoprim and sulfamethoxazole for otitis media [6,7]. Furthermore, recent theoretical work indicates that synergistic drugs can prevent treatment failure even when bacteria resistant to one of the drugs is present at the beginning of therapy [8].

The principle advocating synergy between antibiotics extends to avoiding combinations that inhibit each other and may prolong the infection. Antagonism, when a drug hinders the effect of another, has been reported early in the history of antibiotics and continues to function as a warning against indeterminate treatment [9]. In a study of pneumococcal meningitis patients, 30% of those treated with penicillin alone failed treatment and died, while 79% of comparable patients who were treated with the same dosage of penicillin as well as chlortetracycline died [10,11]. An increasing number of laboratory studies, however, indicate that antagonistic drug combinations as clinical options merit more investigation [12].

While the effects of drug interactions on treatment outcome are well documented, recent work in this area suggests that the different types of interactions have significant effects on the selection and maintenance of drug resistance mutations. Using a direct competition experiment, Chait and colleagues demonstrate how a hyper-antagonistic drug combination is able to select against a bacterial population resistant to one of the drugs and instead favor the completely sensitive partner [13]. Aside from influencing competition, antagonistic drug combinations may even delay the evolution of resistance. The rate of adaptation of laboratory bacteria to multiple drugs has been shown to correlate with the degree of synergism between individual antibiotics [14]. Although antagonistic drug combinations are currently eschewed in clinical set-

tings, these studies suggest that antagonism between antibiotics may aid in devising treatment strategies specifically aimed at delaying the emergence of resistance.

In response to the slow development of new antimicrobials, there is renewed interest in old drugs that have fallen out of use due to toxicity or drawbacks in efficacy [15]. One suggestion that could be implemented to return these drugs to the clinic is to use an old drug in conjunction with a current drug [16]. The advantages of synergism and the diverse, non-trivial effects of antagonism will play a central role in determining how best to implement combination therapy in clinical settings.

In order to exploit the potential benefits of combination therapy we need a better understanding of the circumstances under which synergism versus antagonism is expected. Determining how a broader spectrum of drugs interact at inhibitory concentrations and delineating the mechanisms responsible for these effects could allow for a more prudent application of antibiotics that maintains clinical capability and does not sacrifice the future utility of these drugs.

In this study, we ask whether a basic pharmacodynamic property of all antibiotics can help predict which pairs would result in antagonism. If bactericidal drugs are most potent on actively growing cells, then the inhibition of growth induced by a bacteriostatic drug should result in a reduction of drug efficacy [17,18]. To test this hypothesis, we scour the topology of antibiotic interactions by employing screening methods to identify effects across pairs of 21 different drugs at sub-inhibitory concentrations. We then take a step towards increased clinical relevance by verifying a subset of pairs of interacting drugs at inhibitory drug concentrations as time-kill curves. Since our hypothesis relies on the decreased antibiotic susceptibility of slowly growing cells and the ability of some drugs to influence this state, we repeated our experiments on the level of individual cells using time-lapse microscopy in microfluidic devices to investigate the cellular dynamics underlying combined effects of antibiotics.

# Results

### Systematic exploration of interactions between bactericidal and bacteriostatic antibiotics

To quantify interactions between antibiotics, we first selected 21 antibacterial compounds. These antibiotics cover a wide range of mechanisms of action and many of them are widely employed in the clinics. Their main targets include DNA synthesis, translational machinery, cell wall, folic acid, and lipid biosynthesis (Table S1). We systematically measured the effects of all pairs of antibiotics on growth rates of *E.coli* K12. Specifically, for each pair of antibiotics, we combined 6 different concentrations of the agents in a two-dimensional

dose-matrix with dose points being centred on each antibiotic's half-maximal effective concentration ($EC_{50}$). We considered the Loewe additivity model to assess interactions between antibiotics [19]. In contrast to other models, it measures drug-drug interactions based on deviation from a drug-with-itself reference. According to the Loewe additivity model, if the modes of action of two drugs are the same, they show no interaction. Lines of equal effective dosage (isoboles) are represented in the two-dimensional linear concentration space of the two drugs. Deviation of these lines from a linear model indicates synergism (concave) or antagonism (convex). We introduced several correction steps to overcome potential measurement biases due to plate inhomogeneity, and also developed a rigorous statistical framework to assess the significance of interactions. Following a previously developed method, we quantified isobole shapes by measuring concavity (B), where B=0 indicates independent effects of the two drugs (linearity), while B<0 and B>0 indicate antagonism and synergism, respectively (see Methods). In this work, we focus on studying the properties of antagonistic antibiotic pairs only, while synergism will be studied elsewhere.

Using a statistical criterion to identify significant interactions (see Methods), we found that 61% of the 204 antibiotic pairs showed antagonism. The reliability of our results was confirmed by comparing to a previous systematic drug combination screen performed in *E. coli* [20]. In spite of the substantial differences in the protocols and the underlying assumptions of the models used in the two studies, interaction scores are well correlated (Spearman's rho=0.529, P<0.0001) and antagonistic pairs identified in the previous study tend to have especially low scores in our screen (Mann-Whitney U-test, P<0.0001). Furthermore, our high-throughput survey correctly identified a well-characterized antagonism between ciprofloxacin and tetracycline [21].

The distribution of antagonism is highly non-random on the map of interactions. In line with expectations of the Loewe model, antibiotic pairs that target the same cellular subsystem rarely show strong antagonism (P<0.0001). More strikingly, combinations of 30S protein synthesis and cell-wall biosynthesis inhibitors, 50S protein synthesis and gyrase inhibitors and cell-wall biosynthesis and folic acid synthesis inhibitors frequently showed antagonism (P<0.05 for each combination, Fisher's exact test). A common property of these combinations is that a bacteriostatic compound is combined with a bactericidal agent. In contrast to bacteriostatic antibiotics that slow or inhibit bacterial growth without causing a substantial short-term population decline, bactericidal antimicrobial compounds actively kill bacteria. To minimise the chance of erroneous classification, we used only *E. coli*-specific information for bactericidal/bacteriostatic classification (Table S1). To more generally test whether antagonism is enriched in bacteriostatic-bactericidal combinations, we categorised antibiotic pairs according to individual antibiotic killing properties, leading to three major groups (cid-cid, cid-stat and stat-stat pairs). Strikingly, we found

that antibiotic pairs with exceptionally low B interaction scores are nearly always bactericidal-bacteriostatic (Figure 1, P=0.0016, Fisher's exact test). This result also holds after excluding antibiotic pairs with overlapping cellular targets (P=0.0059). Thus, our data suggests that bacteriostatic agents antagonize the action of antibiotics acting on growing cells. This finding is broadly consistent with earlier reports demonstrating that growth inhibition via nutrient limitation often diminishes the effect of bactericidal compounds [22,23]

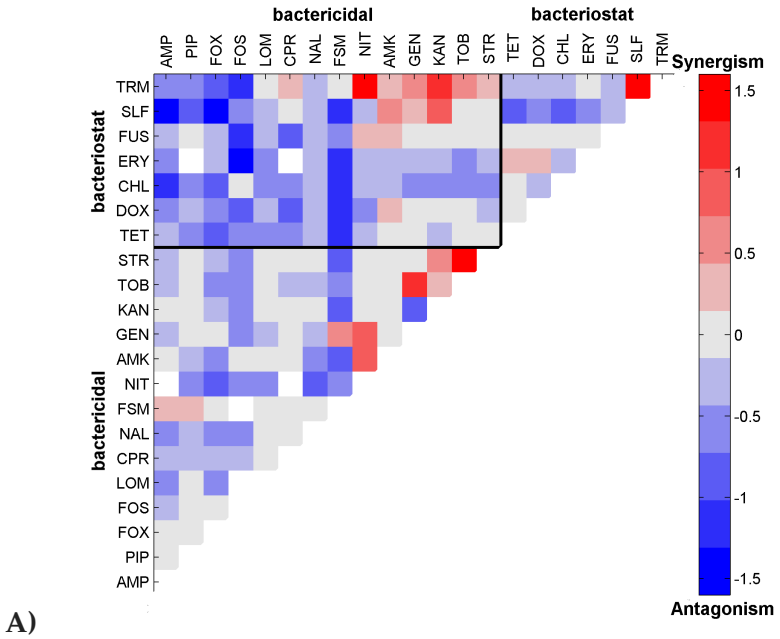



**Figure 1. Systematic exploration of interactions between bactericidal and bacteriostat antibiotics**

**A)** Heatmap showing pairwise interactions systematically measured between 21 antibiotics in *E. coli*. Antibiotics are grouped according to their mode of action (see Table 1) and colors reflect the interaction scores between them. Negative and positive scores correspond, respectively, to antagonism (blue) and synergism (red) according to Loewe additivity criteria. Grey indicates missing data.

**B)** Combining a bacteriostatic with a bactericidal antibiotic has a tendency to show strong antagonism. Antibiotic pairs were categorized according to their individual antibiotic killing properties leading to three major groups: cid-stat, stat-stat, and cid-cid. Antibiotic pairs with lower than -1 interaction scores (I,e, those showing strong antagonism) are significantly more likely to fall in the bactericidal-bacteriostatic category than the rest of antibiotic

**Time-kill curves**

The screen for antagonistic and synergistic pairwise interactions was based on measuring growth rates, and was thus performed at sub-inhibitory concentrations of the antibiotics. To test whether the results of the screen could be extrapolated to clinically relevant drug concentrations, we measured death rates at inhibitory drug concentrations based on time-kill curves. We tested every possible bactericidal-bacteriostatic pair among five antibiotics across a range of concentrations from half to twice their minimum inhibitory concentration or MIC (Table S2). The antibiotics used in the time-kill experiments were selected for their differing mechanisms of action and because to our best knowledge, there are no reports of cross-resistance mutations for these drugs in *E. coli* .

In the presence of a bactericidal drug that alone is capable of clearing a bacterial population, the addition of a bacteriostatic drug resulted in a decrease in killing rates and a significant number of survivors at the end of the experiment (Figure 2). We also noted that the degree of antagonism differs depending on the bactericidal drug employed in the experiment. Acquisition of a resistance mutation over the course of the time-kill curve experiment could be a confounding effect explaining the reduced death rate observed in bacterial cultures treated with a bactericidal-bacteriostatic pair. In order to control for this possibility, the colonies obtained at the end of every time-kill curve experiment were replica-plated on antibiotic-containing plates. We found no evidence for the evolution of single or multidrug resistance in any replicate to any of the drugs used in the experiment (data not shown).

These time-kill curves provide confirmation that the antagonistic interaction found between drugs at concentrations below the MIC extend to concentrations that rise to inhibitory levels. Furthermore, this interaction manifests as a decrease in the rate of killing and the presence of a significant proportion of sensitive bacteria at the end of the experiment.

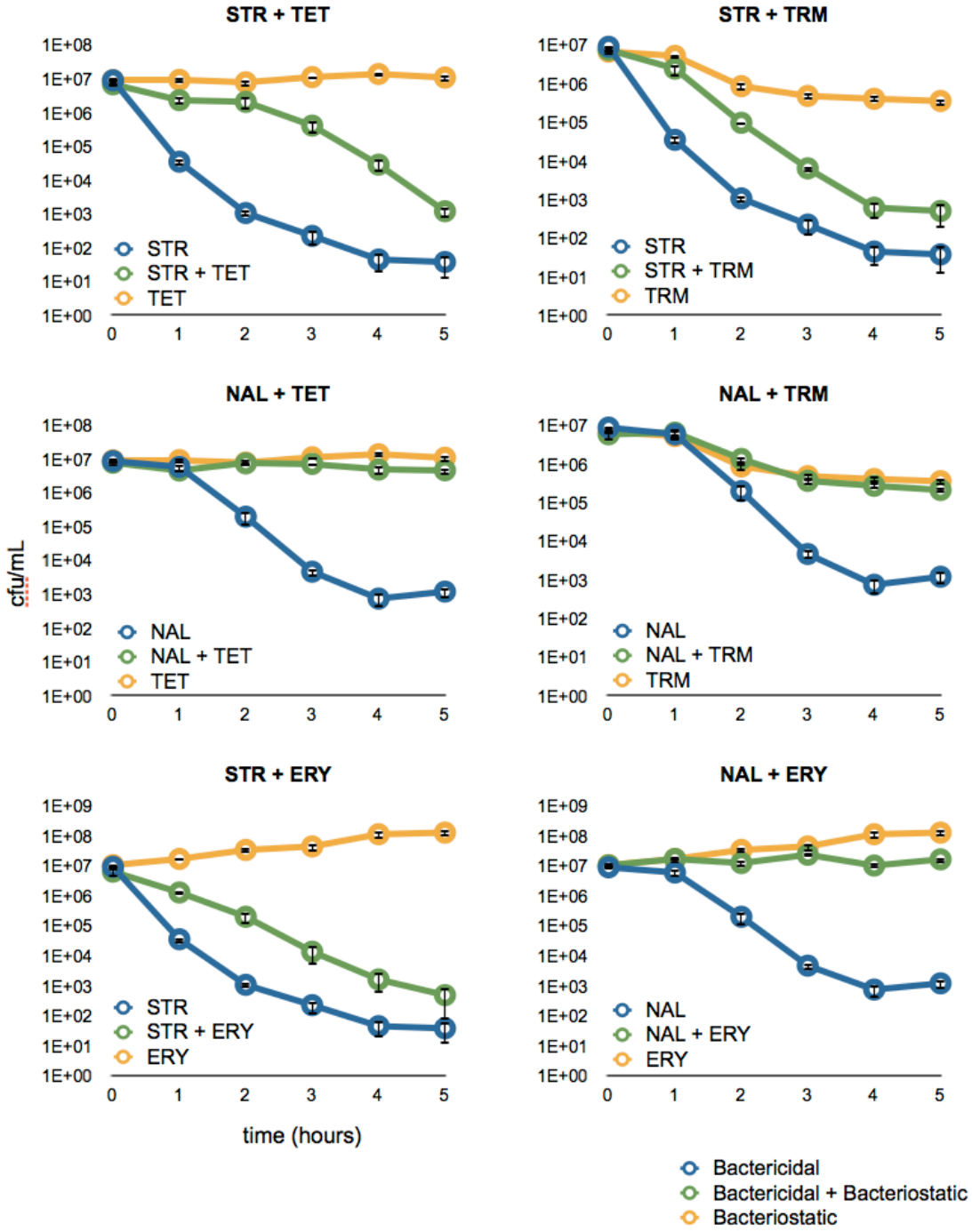



**Figure 2. Time-kill curves for single and two-drug combinations.**

Each graph shows the time-kill curve for a bactericidal/bacteriostatic drug pair and the constituent individual drugs. Error bars represent standard errors of the mean for the number of culturable cells, as measured in colony-forming units (cfu/mL) at each time point.

**Single and combination therapy from the perspective of single cells**

We replicated the conditions of our time-kill curves in a microfluidic device with the goal of directly observing the effects of different antagonistic drug pairs on single cells. Using a device designed to track the elongation and division of hundreds of individuals over a long period of time [24], we began by growing *E. coli* cells in rich media without antibiotics for at least four hours to determine baseline rates of these cellular processes. This was followed by exposure to a single drug or pairs of drugs for at least 16 hours. Media were then switched back to drug-free broth for at least four more hours.

By analyzing the resulting time-lapse images, we captured quantitative information on the rates of cell elongation and division of single cells as they were subjected to different antibiotics singly or in antagonistic pairs. Furthermore, we followed the fates of hundreds of cells from every condition for viability after withdrawal of antibiotic-containing media. While every cell exposed to bactericidal drugs either lysed or did not resume growth by the end of the experiment, at least 30% and up to more than 80% of individuals treated with bactericidal-bacteriostatic drug pairs maintained cellular integrity and resumed division after replacement with antibiotic-free media (Table S3).

We selected at least 20 individual cells per condition for detailed analysis using custom software. Figure 3 depicts the lengths of these cells for each condition across the entire duration of the experiment. The graphs reveal qualitative differences in cellular responses to different antibiotic treatments. Our time-lapse images revealed contrasting responses to the two bacteriostatic drugs tetracycline and erythromycin. As previously reported, we found that tetracycline exposure reduced cell elongation and decreased the rate of cell division [21]. Erythromycin, however, only reduced the rate of division resulting in filamentous cells that continue to divide. The maximum length depicted in our plots is constrained by the length of the growth channel in the microfluidic device – we are thus unable to measure the actual size of antibiotic-induced filaments.

In addition to tracking the rate of elongation of cells in the selected conditions, we also extracted the rates of division of each individual cell as they are subjected to different antibiotic-containing media. In Figure 4 we present the distributions of the division rates and elongation rates (as cell length doublings per hour) for each population in the period of exposure to antibiotics. In order to facilitate comparison, data from all cells grown in rich media prior to the addition of antibiotic is pooled and presented as the “no antibiotic” condition. The dashed line on this graph represents the combination of division and elongation rates needed to maintain a constant size. Cells to the right of this line, therefore, continuously increase in size. This is evident in cells exposed to the two single bactericidal antibiotics, nalidixic acid and streptomycin. These drugs primarily decreased division rates but did not strongly affect elongation. In contrast, the

single bacteriostatic antibiotics and the paired drugs result in large reductions in elongation rate.

The differences in cellular responses observed in the study of cells exposed to bacteriostatic drugs extend to the results for antagonistic drug pairs. Cells treated with tetracycline and either of the bactericidal drugs greatly reduced their growth rate and ceased dividing until the antibiotics were withdrawn while erythromycin paired with a bactericidal drug halted cell division resulting in long filamentous cells that only proceeded with division after replacement with drug-free media. Our time-lapse microscopy experiments thus reveal contrasting responses of bacteria exposed to drugs belonging to the same pharmacodynamics class. These differing responses extend to treatment with different antagonistic drug pairs.

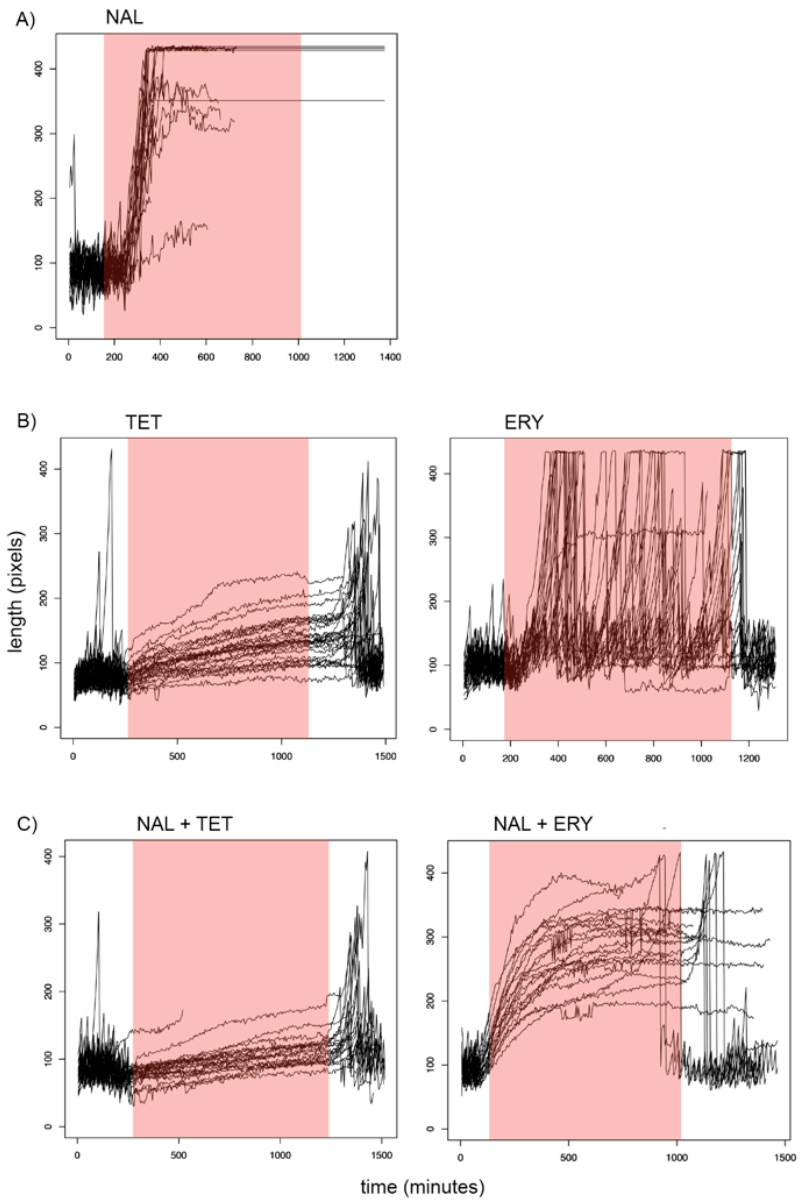



**Figure 3. Length-time graphs of individual cells pre/peri/post-antibiotic exposure.**

At least 20 individual cells per antibiotic treatment were selected for analysis using custom software; the results of each treatment are collated in one graph with each line representing the length of a single cell over time. Upward deflections of these lines, therefore, denote increases in length and abrupt downward deflections are division events. This is corroborated with manual inspection of the resulting time-lapse movies. The highlighted section in each graph denotes the period in which antibiotic-containing media is used. Lines that end abruptly are due to lysis of the cell under observation. The analysis of a population subjected to erythromycin alone was problematic due to the formation of long filamentous cells that are drawn out of their growth channels as media flows through the primary trench of the device – this reduced the number of cells available for observation over the entire experiment. Furthermore, note that in the erythromycin sub-figure, the large fluctuations of cell length denote the increased size to which these cells were observed to grow as well as the continuation of division events.

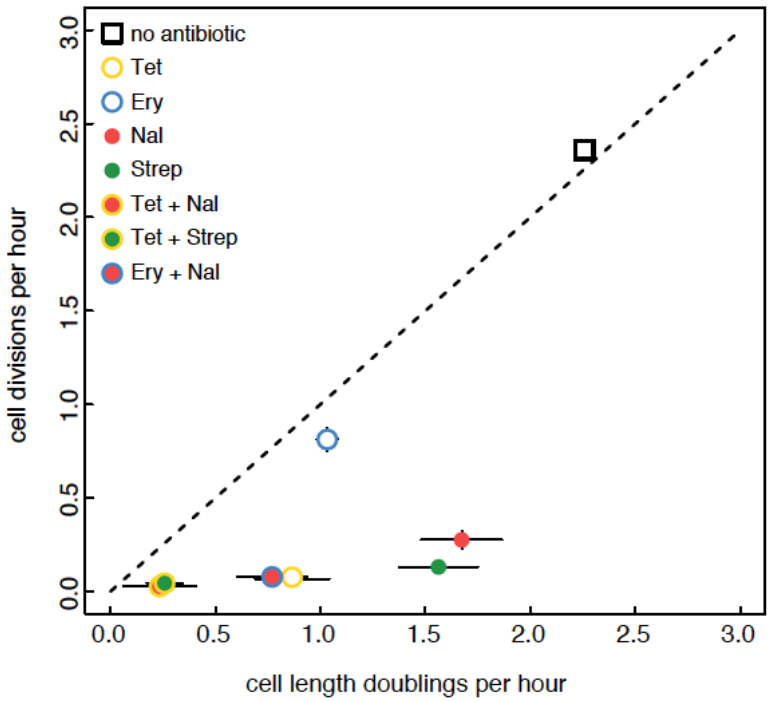



**Figure 4. The effects of single and paired antibiotics on cell division and elongation.**

We extracted the rates of division and elongation from each of the individual cells included in our analysis. Error bars denote the standard errors of the mean. The 45-degree line in this graph represents the combination of division rate and elongation rate (as doublings of cell length) needed to maintain a constant cell size.

# Discussion

The relevance of classifying antibiotics as bacteriostatic or bactericidal has been questioned due to the reliance of these categories on concentration as well as on the organism to which it is applied [25]. The manner in which these pharmacodynamics properties are used in specific clinical scenarios is beyond the scope of this paper. Instead we propose that this binary classification is a useful initial step in determining when two drugs in combination would result in antagonism and thus should be evaluated to exploit the varied effects of this specific interaction.

The tests set by the Clinical and Laboratory Standards Institute [26] to determine whether an antibiotic is bacteriostatic or bactericidal involve assessing the degree of survival of a liquid culture of bacteria after a certain period of drug exposure. The moderate killing effect that defines a bacteriostatic agent therefore implies an induction of cellular stasis. Here we show that while bacteriostatic drugs result in prevalent patterns of antagonistic interactions with bactericidal drugs, their effects on the single cell level may differ considerably. We

found that tetracycline effectively induces stasis in antibiotic-sensitive bacteria. We observed this drastic reduction in growth rate and complete arrest of division for the entire period of exposure to sub-inhibitory tetracycline. In stark contrast, treatment with erythromycin only reduced elongation rate to a similar degree as tetracycline however division rates were not as strongly decreased; this resulted in long, filamentous cells.

The differences in morphological response to similarly antagonistic antibiotic combinations (Figure 3C) suggest that different cellular mechanisms are underlying the increased survival in these pairs of drugs. However, we found that adding two different bacteriostatic drugs to a bactericidal drug both result in decreased elongation rates. This reduction in rates of elongation could be the basis of antagonism. Further work is needed to rule out other potential arbiters of this interaction, however our results suggest that the relationship between antibiotic killing and cell growth should be disentangled further to consider division and elongation separately. Determining conditions that specifically affect elongation, therefore, could be important in designing strategies that increase the efficiency of antibiotic-mediated killing.

Our results urge caution before forming general assumptions on the effects of drug interactions. Although our studies of bacteriostatic and bactericidal drugs reveal pervasive antagonism on growth and death rates, the variety in morphological responses we observe may lead to antibiotic combination-specific fitness effects. Furthermore the bacteriostatic/bactericidal classification system will vary across organisms and the interactions between drugs may similarly shift. Antibiotic combination therapy remains an important viable option as a treatment strategy aimed at controlling the rise of resistance. As this goal is approached, the single cell dynamics we observed under different antagonistic drug pairs highlight the importance of multiple experimental perspectives to increase our understanding and predictive power in utilizing drug combinations for more bacterial infections.

# Methods

### High-throughput combination screening experiments

We selected 21 antibiotics that cover a wide range of mechanisms of action, including drugs that target cell wall, nucleic acid, protein and folic acid biosynthesis (Table 1). Fresh antibiotic solutions were prepared from powder stocks on a weekly basis and filter-sterilized before use. All experiments were conducted in *Escherichia coli* K12 (BW25113) in minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. Combination screens were performed in 384-well plates using a robotic liquid handling system (Hamilton Star Workstation) to improve reproducibility. Culture volume was 50 $\mu$l. Each plate contained two different 6 x 6 dose-matrix blocks (one antibiotic in combination

with two other antibiotics) in 4 replicates each. For each pair of antibiotics, we combined 6 different concentrations of the agents in a serially diluted two-dimensional dose-matrix with dose points being centred on each antibiotic's half-maximal effective concentration ($EC_{50}$). The lowest concentration for each agent was 0 and the highest was above $EC_{90}$ (see Table 1). In addition to the dose-matrix blocks, each plate included 18 wells containing a medium devoid of antibiotics (control wells).

Antibiotic sensitivity screens were performed by growing cells overnight (OD600=4) at 30°C shaken at 300 rpm, diluting until they had an OD equivalent to 0.04. Next, using liquid handling robotics, cells were transferred into 384-well assay plates (in the presence of antibiotics) to yield $4x10^4$ cells/well. Assay plates were incubated at 30°C shaken at 300 rpm for 18h. Bacterial growth was monitored by measuring optical density (OD 600) of the liquid cultures at a single time point. Preliminary experiments showed that a single reading of optical density after 18 hours of incubation shows strong linear correlation with the area under the growth curve, a descriptor of overall inhibitory effect that covers the entire growth period [27]. Plates were prepared in multiple (up to 6) biological replicates and those with quality control issues were omitted from further analysis (i.e. growth of control wells was unusually low or showed large variations, an agent failed to substantially inhibit growth at high concentrations or strongly inhibited growth at even low doses).

**Data processing and bias correction steps**

To overcome any measurement bias caused by within-plate inhomogeneity, we processed the raw optical density data as follows. We included 18 control wells on each plate containing a medium devoid of antibiotics and inoculated by *E. coli*. We used these wells both to set a baseline for zero inhibition and to estimate and eliminate within-plate systematic biases. First, we calibrated OD values by applying the transformation $OD_{calibrated} = OD + 0.40449*OD^3$ to account for the non-linear association between OD and cell density at high cell densities (parameters of the calibration formula were derived as in ref [28]). Then we calculated relative inhibition values based on the initial OD (maximum inhibition) and the average OD of antibiotic-free control wells (maximum growth). To estimate and eliminate within-plate spatial effects, we first fitted a linear trend to the control wells to eliminate spatial gradients. Next, for the residuals, we employed Gaussian process regression [29] to eliminate the remaining systematic spatial biases using the control wells. Both bias correction steps were carried out in MATLAB.

**Identifying interacting antibiotic pairs**

To assess antagonism and synergy between pairs of antibiotics we used the Loewe additivity model [30], which assumes that a drug does not interact with itself. Geometrically, Loewe additivity can be represented as lines of equal effective dosage (isoboles) in the two-dimensional linear concentration space of the two drugs. Deviation of the shape of isoboles from linearity indicates either synergy (concave isoboles) or antagonism (convex isoboles). To identify interactions for each pair of antibiotics, we first merged data from replicate dose-matrix blocks located on the same 384-well plates. Next, we fitted sigmoidal dose response curves (Hill equation) to the single agent responses using a maximum likelihood fitting procedure. Based on the single agent response curves of the two antibiotics, we calculated the dose response of the combined antibiotics expected under the Loewe additivity model. To quantify interactions, we determined the concavity of the set of isoboles inferred from the combination measurements for a given antibiotic pair ('observed' isoboles). To achieve this, we used a mathematical transformation to 'bend' the linear isoboles expected under the Loewe additivity model to most closely approximate the observed isoboles (see Cokol et al. for a similar approach [31]). The transformation relies on a single parameter to describe the concavity of the observed isoboles, which we use as a measure of antibiotic interaction. This score is zero in the absence of interaction, negative for antagonistic and positive for synergistic pairs. Finally, interaction score for each antibiotic pair was calculated by taking the median score obtained from biological replicates (i.e. independent plates).
Measurement error of interaction screens was estimated by testing 5 antibiotics for interactions with itself in multiple replicates [31]. Because under Loewe additivity a drug shows no interaction with itself, deviation of the interaction score from zero provides an estimate of the experimental error of interaction measurements. Thus, we considered two antibiotics as significantly interacting when their score was significantly different from the mean score of self-self antibiotic combinations.

**Time-Kill Curves**

Overnight cultures of *E. coli* MG1655 were diluted 1:10,000 into fresh, pre-warmed LB broth and incubated for 2 hours. A further 1:2 dilution was performed before introduction into flasks containing either a single or a pair of bactericidal and bacteriostatic antibiotics. These were then incubated with shaking and aeration at 37°C. Samples were taken at 1-hour intervals for up to 5 hours. Cell densities for each sample were estimated from colony counts by dilution in phosphate-buffered saline and plating on LB agar. Each time-kill experiment was performed twice.

**Time-Lapse Microscopy**

Specific details of the microfluidic system used in this study, the mother machine, have been described previously [32]. In brief, this device consists of 4000 growth channels arranged at right angles against a large trench through which growth media is passaged. Nutrients then diffuse into the channels and flush out growing cells as they emerge from these channels. An automated microscope stage allows for the monitoring of multiple fields of view spanning the entire device. This method results in the continuous observation of growth and division of a large number of individual cells as they experience different antibiotic-containing environments and their survival or death after drug has been removed.

Time-lapse microscopy experiments were conducted as follows: *E. coli* MG1655 cultures were grown overnight in LB at 37°C. On the following day, 100 $\mu$L of this culture was diluted in 10 mL of fresh, pre-warmed LB and was then incubated with shaking at 37°C for 2 hours. 8 mL of the resulting culture was spun down and resuspended in 20 $\mu$L of fresh LB. 10 $\mu$L of the cell suspension was then injected into the mother machine; the experiment was initiated when more than 80% of the channels were filled with cells via diffusion. A syringe pump was used to passage fresh LB supplemented with bovine serum albumin (BSA) and salmon sperm DNA through the device at a rate of 2 mL/h. Images were acquired from 15-25 fields of view at 6 min intervals via an automated Olympus BX81 microscope with a UPLFN100xO2PH/1.3 phase-contrast oil lens. Samples and the microscope were held at 37°C with a Cube and Box incubation system (Life Imaging Services, Reinach, Switzerland). After at least 4 hours of growth in LB, the media was switched to LB containing BSA, salmon sperm, and either single or two antibiotics. Cells were exposed to this regimen for at least 20h before being switched back to fresh LB supplemented with BSA and salmon sperm DNA for up to 10h. Each experiment consists of fields scanned continuously for at least 30h.

The resulting time-lapse images were then analyzed with a custom-designed plugin for ImageJ to provide information on cell size and division rates during the three different phases of the experiment (available at http://projects.exeter.ac.uk/ein/mmj/doku.php). The first step of the analysis consists of defining the length of the cell abutting the end of the channel. The increasing length of the growing cell over succeeding frames is tracked and recorded; division events are also registered based on cell length. Manual verification and annotation were performed after every experiment. In this way, we were able to extract quantitative information on an individual cell's elongation and division rates. We also tracked the proportion of cells that survived treatment exposure and were able to divide again upon the return to an antibiotic-free environment. The occurrence of filamentation during exposure to the antibiotics erythromycin and nalidixic acid lead to elongated cells being pulled out of their growth channels during flow. For this reason, only channels containing

cells that could be followed for the entirety of the experiment were considered in the analysis.

**Author contributions**

MA, SB, CP, and PSO conceived and designed the experiments. VL and BB performed the pairwise screen of antibiotic interactions. VL and BB analyzed the results of the screen, and with CP wrote the corresponding Results and Methods section. PSO performed the time-kill curve and microfluidics experiments. PSO, PAzW, M Arnoldini, and SB analyzed the microfluidics data. PSO wrote all other sections of the paper. All authors discussed the results and implications and commented on the manuscript.

# References


1. Bonhoeffer S, Lipsitch M, Levin BR (1997) Evaluating treatment protocols to prevent antibiotic resistance. Proc Natl Acad Sci USA 94: 12106–12111.
2. Hand K (2006) Tuberculosis: Pharmacological Management. Hospital Pharmacist 13: 81–85.
3. Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, et al. (1998) Changing patterns of mortality across Europe in patients infected with HIV-1. Lancet 352: 1725–1730.
4. WHO (2010) WHO Guidelines for the treatment of malaria, Second edition.
5. Cottarel G, Wierzbowski J (2007) Combination drugs , an emerging option for antibacterial therapy. Trends Biotechnol 25: 547–555. doi:10.1016/j.tibtech.2007.09.004.
6. Forrest GN, Tamura K (2010) Rifampin combination therapy for nonmycobacterial infections. Clin Microbiol Rev 23: 14–34. doi:10.1128/CMR.00034-09.
7. Cunha BA (2004) Therapeutic implications of antibacterial resistance in community-acquired respiratory tract infections in children. Infection 32: 98–108. doi:10.1007/s15010-004-3065-5.
8. Ankomah P, Johnson PJT, Levin BR (2013) The Pharmaco –, Population and Evolutionary Dynamics of Multi-drug Therapy: Experiments with S. aureus and E. coli and Computer Simulations. PLoS Pathog 9: e1003300. doi:10.1371/journal.ppat.1003300.
9. Johansen HK, Jensen TG, Dessau RB, Lundgren B, Frimodt-Moller N (2000) Antagonism between penicillin and erythromycin against Streptococcus pneumoniae in vitro and in vivo. J Antimicrob Chemother 46: 973–980.
10. Cates J, Christie R, Garrod L (1951) Penicillin-resistant Bacterial Endocarditis Treated by Penicillin and Streptomycin. Brit Med J 1: 656–656.
11. Garrod L (1972) Causes Of Failure In Antibiotic Treatment Garr. Brit Med J 4: 473–476.

12. Yeh PJ, Hegreness MJ, Aiden AP, Kishony R (2009) Drug interactions and the evolution of antibiotic resistance. Nat Rev Microbiol 7: 460–466. doi:10.1038/nrmicro2133.

13. Chait R, Craney A, Kishony R (2007) Antibiotic interactions that select against resistance. Nature 446: 668–671. doi:10.1038/nature05685.

14. Hegreness M, Shoresh N, Damian D, Hartl D, Kishony R (2008) Accelerated evolution of resistance in multidrug environments. Proc Natl Acad Sci USA 105: 13977–13981. doi:10.1073/pnas.0805965105.

15. Nitzan O, Supornitzky U, Kennes Y, Chazan B, Raz R, et al. (2010) Is chloramphenicol making a comeback? IMAJ 12: 371–374.

16. Falagas ME, Grammatikos AP, Michalopoulos A (2008) Potential of old-generation antibiotics to address current need for new antibiotics. Expert Rev Anti Infect Ther 6: 593–600. doi:10.1586/14787210.6.5.593.

17. Jawetz E, Gunnison J (1953) Antibiotic synergism and antagonism: an assessment of the problem. Pharmacol Rev 5: 175–192.

18. Jawetz E, Gunnison J, Coleman VR (1954) Observations on the Mode of Action of Antibiotic Synergism and Antagonism. J Gen Microbiol 10: 191–198.

19. Loewe S (1928) Die quantitiven Probleme der Pharmakologie. Ergebnisse der Physiologie 27: 47–187.

20. Yeh P, Tschumi AI, Kishony R (2006) Functional classification of drugs by properties of their pairwise interactions. Nature Genetics 38: 489–494. doi:10.1038/ng1755.

21. Bollenbach T, Quan S, Chait R, Kishony R (2009) Nonoptimal microbial response to antibiotics underlies suppressive drug interactions. Cell 139: 707–718. doi:10.1016/j.cell.2009.10.025.

22. Cozens RM, Tuomanen E, Tosch W, Zak O, Suter J, et al. (1986) Evaluation of the bactericidal activity of beta-lactam antibiotics on slowly growing bacteria cultured in the chemostat. Antimicrob Agents Chemother 29: 797–802. doi:10.1128/AAC.29.5.797.Updated.

23. Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE (1991) Bactericidal Effects of Antibiotics on Slowly Growing and

Nongrowing Bacteria. Antimicrob Agents Chemother 35: 1824–1828. doi:10.1128/AAC.35.9.1824.Updated.

24. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, et al. (2010) Robust Growth of Escherichia coli. Curr Biol 20: 1099–1103. doi:10.1016/j.cub.2010.04.045.

25. Pankey G, Sabath L (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clin Infect Dis 38: 864–870.

26. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Seventh Edition. Clinical Laboratory and Standards Institute (2006)

27. Pena-Miller R, Laehnemann D, Jansen G, Fuentes-Hernandez A, Rosenstiel P, et al. (2013) When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. PLoS Biol 11: e1001540. doi:10.1371/journal.pbio.1001540.

28. Warringer J, Blomberg A (2003) Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in Saccharomyces cerevisiae. Yeast 20: 53–67. doi:10.1002/yea.931.

29. Carl Edward R, Williams CK (2006) Gaussian processes for machine learning. Cambridge, MA: MIT Press.

30. Loewe S (1953) The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3: 285–290.

31. Cokol M, Chua HN, Tasan M, Mutlu B, Weinstein ZB, et al. (2011) Systematic exploration of synergistic drug pairs. Mol Sys Biol 7: 544. doi:10.1038/msb.2011.71.

32. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, et al. (2010) Report Robust Growth of Escherichia coli. Curr Biol 20: 1099–1103. doi:10.1016/j.cub.2010.04.045.

Table S1. List of all antibiotics used in the study

| Drug abbreviation | Antibiotic | EC90 (μg/ml) | Main mechanism action | Bactericidal or Bacteriostatic |
|---|---|---|---|---|
| AMP | ampicillin | 1.2 | cell wall | cid |
| PIP | piperacillin | 1.5 | cell wall | cid |
| FOX | cefoxitin | 1.4 | cell wall | cid |
| FOS | fosfomycin | 14 | cell wall | cid |
| LOM | lomefloxacin | 0.12 | gyrase | cid |
| CPR | ciprofloxacin | 0.0055 | gyrase | cid |
| NAL | nalidixic acid | 2.3 | gyrase | cid |
| FSM | fosmydomicin | 40 | lipid | cid |
| NIT | nitrofurantoin | 2.4 | multiple mechanisms | cid |
| AMK | amikacin | 3.4 | aminoglycoside, protein synthesis 30S | cid |
| GEN | gentamicin | 0.66 | aminoglycoside, protein synthesis 30S | cid |
| KAN | kanamycin | 3 | aminoglycoside, protein synthesis 30S | cid |
| TOB | tobramycin | 0.85 | aminoglycoside, protein synthesis 30S | cid |
| STR | streptomycin | 4.5 | aminoglycoside, protein synthesis 30S | cid |
| TET | tetracycline | 0.3 | protein synthesis 30S | stat |
| DOX | doxycycline | 0.23 | protein synthesis 30S | stat |
| CHL | chloramphenicol | 1 | protein synthesis 50S | stat |
| ERY | erythromycin | 8.5 | protein synthesis 50S | stat |
| FUS | fusidic acid | 200 | protein synthesis 50S | stat |
| SLF | sulfamonomethoxine | 1.9 | folic acid biosynthesis | stat |
| TRM | trimethoprim | 0.4 | folic acid biosynthesis | stat |

**Table S2. List of antibiotics used in Time-Kill Curves and Time-Lapse Microscopy**

| Drug abbreviation | Antibiotic | Concentration used (μg/ml) | Bactericidal or Bacteriostatic |
|---|---|---|---|
| NAL | nalidixic acid | 25 | cid |
| STR | streptomycin | 25 | cid |
| TET | tetracycline | 12.5 | stat |
| ERY | erythromycin | 200 | stat |
| TRM | trimethoprim | 10 | stat |

**Table S3. Post-antibiotic treatment individual survival rates**

| Drug condition | Individuals observed over entire experiment | % survival |
|---|---|---|
| NAL | 291 | 0 |
| STR | 318 | 0 |
| TET | 287 | 87.8 |
| NAL + TET | 368 | 83.9 |
| NAL + ERY | 423 | 30.9 |
| STR + TET | 361 | 38.5 |

*Comparable single cell survival rates in erythromycin are unobtainable due to the filamentation induced by the drug. A majority of filamentous individuals are pulled by the flow of media leaving only a few cells remaining in a growth channel for the entire duration of the experiment.

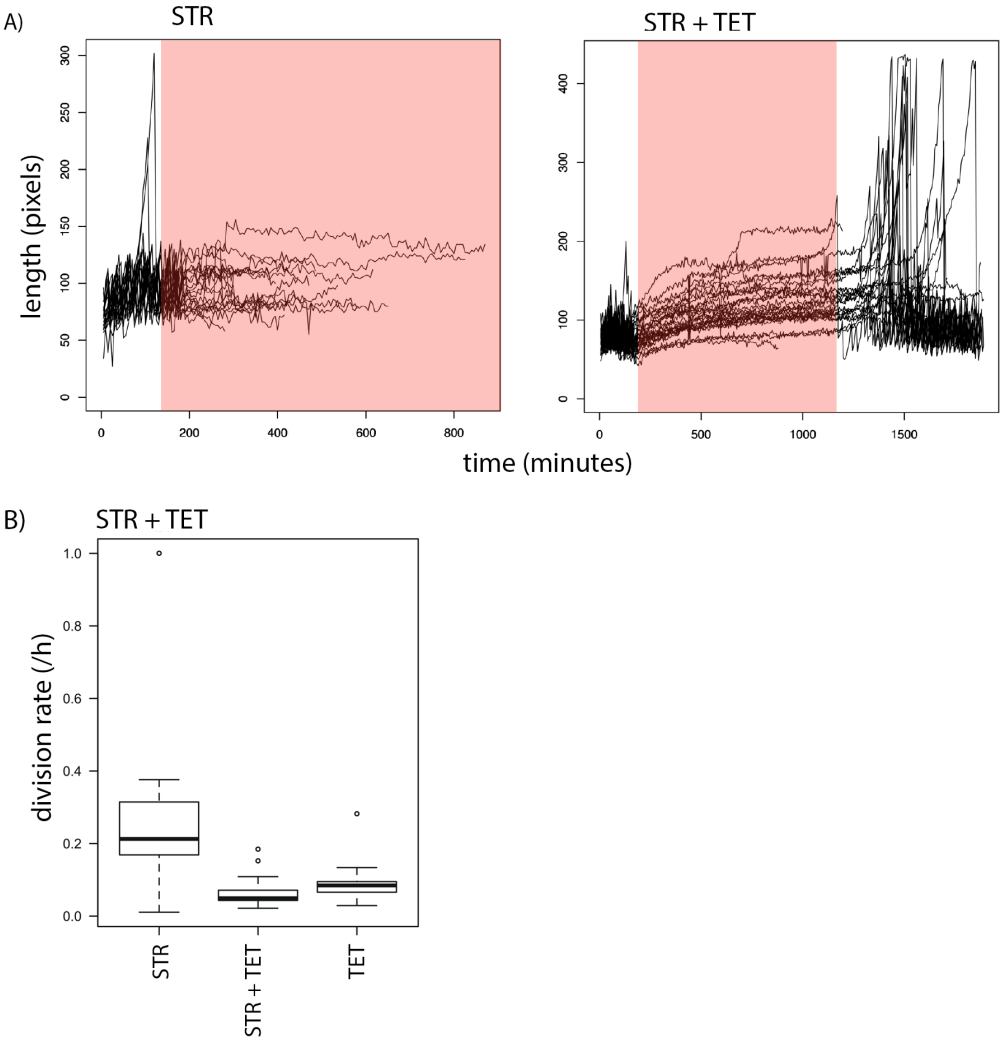



**Figure S1. Length-time graphs of individual cells exposed to streptomycin, and streptomycin + tetracycline.**

A) All cells exposed to streptomycin were observed to die during treatment. In contrast, a significant number of cells exposed to both streptomycin and tetracycline survived antibiotic exposure. B) These boxplots display the distribution of division rates of the cells as they are exposed to either streptomycin, streptomycin and tetracycline, or tetracycline. The division rates of cells in streptomycin and tetracycline are not significantly different from the division rates in tetracycline alone.

# 5

# Discussion

The problem of antibiotic resistance has evolved from isolated individual cases of treatment failure to an insidious epidemic whose scope and magnitude are steadily increasing. The challenge is not just to address the needs of each patient but also to determine the environmental factors that influence clinical resistance, and to design therapies that delay the evolution of resistance.

In this dissertation, I argue that the broadening of research focus to include the period in which bacteria are exposed to antibiotics provides a new perspective from which different aspects of this problem could be addressed. Here, I expand on issues highlighted by my previous research chapters and discuss future directions that may help control the spread of clinical resistance. Specifically, I draw attention to research foci that pertain to how resistance is maintained and spread, and suggest how some bacterial responses to antibiotics could be vulnerabilities that can be exploited. In a final section, I draw on a recurring theme across the experimental results I report to propose an alternative perspective on how the physiological state of antibiotic resistance can be viewed.

## Antibiotic concentration and population dynamics of sensitive and resistant genotypes

There is increasing evidence suggesting that antibiotics used in hospitals and veterinary clinics are entering the environment and impacting the emergence of clinical resistance [1]. Furthermore, it is now recognized that most of the mechanisms of resistance adapted by pathogenic bacteria have been imported from the environmental resistome via mobile genetic elements [2]. Although evidence is mounting for a link between antibiotic consumption in clinical settings leaking into other areas and environmental bacteria sharing their

resistance genes with pathogens, we can only speculate as to how this link is formed, what parameters are important in maintaining this association, and whether these relationships can be manipulated to favor sensitive over resistant bacteria.

In Chapter 2, we examine a mechanism by which bacteria are thought to commonly reduce the cost of resistance mutations thereby increasing their viability in the absence of drug selection. We help describe when and how compensatory mutations aid the maintenance of resistance by measuring the relationship between antibiotic concentration and bacterial genotype. In order to better predict the population dynamics of resistance emergence, more effort should be invested in determining how other mechanisms that spread or support resistance might vary their effects in relation to different parameters such as number, type, and concentration of antibiotics. Two such candidate mechanisms are plasmids and phage.

Plasmids have long been recognized as an important conveyor of resistance among pathogenic bacteria. These mobile genetic elements are able to freely and quickly disseminate themselves across populations of bacteria, and even across species of bacteria. Besides facilitating the spread of antibiotic resistance, they can also indirectly maintain other virulence traits by encoding mechanisms that maintain the plasmids themselves [3]. These include partition systems that operate akin to mitosis and actively segregate plasmids to each daughter cell during division, and post-segregational killing systems that kill daughters that do not contain a plasmid.

The diverse mechanisms at the disposal of plasmids have given rise to successful, globally disseminated, and increasingly frequent plasmid-mediated multidrug resistance. The NDM-1 plasmid is a recent and important example of this phenomenon. Originally discovered in an isolate of *Klebsiella pneumoniae* from a patient in New Delhi in 2007, this plasmid has since been reported in every continent except Antarctica and South America, and in many species such as *Escherichia coli*, *Shigella boydii, Vibro cholera, Acinetobacter*, *Enterobacteriaceae,* and in 12 *Citrobacter* species [4].

Plasmids have clear, demonstrated effects on the spread of antibiotic resistance. We are, however, unsure of how plasmids acquire resistance genes, what factors influence their acquisition, or even in what environments this occurs. The case of NDM-1 serves as an argument for identifying the parameters that control the dynamics of a mechanism that spreads and maintains resistance, and not just the resistance mechanism itself.

Although phage are recognized as important components in shaping the ecology of bacteria in the environment, the processes by which they modify the human microbiome or human pathogens has not been determined. While some phage can enter specific bacterial species and immediately induce lysis, others lie dormant by integrating themselves either as a plasmid or directly onto their

host's chromosome. Bacteria carrying this second, lysogenic type of phage can make use of new traits carried on the phage genome – such as antibiotic resistance. Furthermore, the ubiquity of phage in nearly all environments makes them particularly suspect in aiding the spread of resistance. Functional metagenomic studies revealed a vast array of antimicrobial resistance genes in soil bacteria, in phages isolated from environmental samples, as well as in human fecal microbiota [5–7]. Lysogenic phage are able to mine this bounty of new genes, grant resistance to new hosts they infect, and paradoxically even share these traits as they lyse their current host and seek new ones in response to stressors such as antibiotics [8].

As an example, the treatment of the most common genetic disease among Caucasians is made complicated by the presence of phage. The ability of phage to shuffle the genomes of a bacterial community and generate chimeras better adapted to their local environment has been implicated as a significant contributor to the morbidity of cystic fibrosis (CF) patients [9]. The thick sputum coating the lungs of patients with CF provide a fertile ground for repeated colonization by many bacterial species; treatment, therefore, involves iterative application of broad-spectrum antibiotics. Accordingly, examination of the viromes isolated from CF patients reveals a significantly more abundant and diverse repertoire of resistance genes compared to the viromes from non-CF patients [10]. It is likely that antibiotic treatment itself aids in the spread of resistance via phage transduction. This provides strong motivation in evaluating the relationship between antibiotic treatment and phage, and how this could be manipulated to affect the population dynamics of sensitive and resistant bacteria.

**Plasticity during antibiotic exposure**

Observations of bacteria undergoing stressors including antibiotic treatment have lead to the recognition of genetic, physiological, and morphological plasticity as adaptive responses. Although these mechanisms pose a different set of challenges when compared to controlling traditional resistance genes or mechanisms, how bacteria respond to antibiotic stress may reveal vulnerabilities that could be exploited to increase the efficacy of a treatment regime.

Clinically relevant resistance is most often associated with mutations that alter the targets of drugs, or dedicated resistance genes such as efflux pumps or enzymes that alter the drugs themselves. These mechanisms are inherently costly since antibiotics are directed at essential genes or functions, or the production of pumps or enzymes dedicated to antibiotics result in metabolic costs. Recent work has shown how bacteria utilize alternative forms of genetic mechanisms to achieve high-level resistance.

Instead of acquiring a resistance mutation or gene, overproduction of existing efflux pumps or drug targets has been shown to similarly circumvent a wide variety of antibiotics [11]. These genes are amplified via tandem genetic

duplications that have been found to occur in all regions of the chromosome. Consequently, this form of resistance presents entirely new challenges. They are difficult to identify since the genetic changes they cause are found at the junctions of duplication points and not directly on the genes of interest. Gene duplications are inherently transient which further complicates their detection and study. In addition, whatever costs gene duplications may incur is easily relieved due to their instability.

Aside from traditional resistance genes, bacterial chromosomes may also harbor cryptic resistance genes. These genes are not obviously associated with resistance since they are either expressed at low levels or only under certain conditions. As an example, poor nutrient conditions resulting in slow growth has been found to induce the cryptic aminoglycoside resistance gene, *aadA*, in *S. enterica* [12]. Since the initiation of this particular type of resistance is contingent on growth conditions, this may have significant clinical implications as bacteria bearing this gene would pass as aminoglycoside-sensitive under conventional susceptibility testing.

Aside from the genomic architecture that allows for duplications or cryptic genes that manifest only under certain conditions, bacteria have at their disposal an arsenal of morphological traits that allow them to thrive in stressful environments. In Chapter 4, we observe the formation of elongated daughters in *E. coli* exposed to some bactericidal antibiotics. Filamentation, or more specifically continued cell growth in the absence of septation, results in organisms that are typically 10-50 times longer than their bacillary counterparts and have multiple chromosomal copies. Surprisingly, filamentation has been observed and implicated as a morphological response resulting in bacterial survival against a variety of environmental stresses: including predation by protists, host effects during infection, and exposure to antibiotics [13].

Considering this phenomenon in relation to antibiotic treatment, there are two examples in which the induction of filamentation resulted in greater survival during antibiotic treatment. First, the beta lactam class, antibiotics that interfere with cell wall biosynthesis, induce the SOS response resulting in filamentation. Since beta lactams kill only actively dividing cells, the prevention of division accompanying filamentation delays antibiotic-mediated killing [14]. Furthermore, the induction of the SOS response augments horizontal transfer of DNA, particularly antibiotic resistance elements [15]. In a second example, the developmental changes accompanying antibiotic exposure of *Burkholderia pseudomallei* has been found to result in unanticipated protection against unrelated antibiotics. In a study linking virulence to antibiotic exposure, Chen and colleagues took normal, non-treated *B. pseudomallei* versus the same strain exposed to three different drugs and compared their ability to lyse immortalized immune cells [16]. They found that the bacteria exposed to drugs at or below the MIC resulted in filaments with decreased virulence. However, the removal of drug permitted the resumption of growth and the production of normal-sized,

bacillary daughter cells. These new cells did not filament during re-exposure to drug, were found to be more resistant to new classes of drugs, and were measured to be as virulent as the untreated control. In this case, while the morphological response of filamentation is transient and limited to the period during which drug is present, the changes induced in the cell are long lasting and provide additional survival advantages.

In Chapter 3, I report a novel parallel mechanism by which bacteria may circumvent antibiotic stress. I find that exposing streptomycin-resistant bacteria to an inhibitory concentration of streptomycin aids the growth of daughter cells in a second, unrelated antibiotic. This effect is dependent on both concentration and time, as serial transfer of a resistant population grown in antibiotic to a drug-free environment does not produce this increased growth effect. Similarly, the induction of filamentation we observe in cells exposed to erythromycin in Chapter 4 could be involved in the survival of these cells in combination treatment. These two transient phenomena are a few among likely many morphological and genetic mechanisms utilized by bacteria to withstand killing by antibiotics. As more of these inducible processes are recognized, the justification increases for a reevaluation of both the therapeutic regimes that may be conducive for these mechanisms, as well as susceptibility testing that may miss their identification.

**Increasing the activity of available antibiotics**

In Chapter 4, we argue that combination therapy, the strategy used in controlling resistance in HIV, tuberculosis, and malaria, should serve to inform the treatment of more common bacterial infections. While rigorous clinical testing is of course required before the standard of care should be altered, we show that a basic pharmacodynamic property of all antibiotics is a useful predictor in deciding which pairs of drugs would result in an antagonistic interaction. Such an interaction has been shown to influence both treatment efficacy and the evolution of resistance [17,18]. As resistant infections have increased in frequency, many kinds of combination treatments have been suggested as new means in tackling pathogens.

**Antibiotic combinations that delay resistance**

While there is an abundance of evidence implicating antagonism between antibiotics as a contributor to treatment failure, recent work on this type of interaction raises the possibility of using combinations of drugs to specifically disfavor resistant bacteria. Ordinarily, singly resistant mutants are expected to have some advantage even in the presence of two drugs. Chait and colleagues suggest that this may not be the case if the two drugs in combination result in an extreme form of antagonism - suppression [19]. This subclass of antagonism is when the combined effect of two drugs is less than at least one of the drugs alone. In such an environment, resistance would remove the effect of one drug

but it would also remove the suppressive interaction. They test their hypothesis by competing sensitive and resistant bacteria in either a synergistic or suppressive two-drug matrix. They find specific regions of suppressive drug concentration combinations that both select against the resistant mutant as well as inhibit the wildtype. This raises the prospect of devising treatment regimes that specifically oppose the evolution of resistance.

**Antibiotic adjuvants**

Since the 1960s, only three novel antibiotic classes have received approval by regulatory agencies, and resistance emerged for one of them even before it was put to use in clinics [20]. The scarcity of new treatment options has prompted a reappraisal of the currently available drugs and a search for creative methods by which to increase their clinical utility. In Chapter 4, we take inspiration from how resistance is prevented in many chronic diseases and argue that using two different antibiotics in combination should be considered in order to achieve the same effect in more common bacterial infections. In addition, old drugs that have fallen out of use due to severe side effects or widespread resistance can also be resurrected by combining it with an adjuvant. This second molecule could potentiate the effect of an antibiotic by modifying a cell's physiology to increase susceptibility, or by increasing the concentration of the antibiotic within a cell.

Although the toxic effects of colistin have limited its use, clinics burdened with an increase of multidrug resistant Gram-negative bacteria (*A. baumanii, P. aeruginose, K. pneumoniae*) have had little choice but to reintroduce the drug. Colistin (polymyxin E) is a cyclic cationic polypeptide antibiotic that permeabilises the outer membrane of Gram-negative bacteria. Recently, scientists have shown how combining colistin with a second antibiotic not normally used against Gram-negative bacteria, such as rifampicin or vancomycin, can produce numerous benefits [21,22]. First, colistin functions in these cases only to permeabilise a cell for entry of the second drug and not for its own bactericidal effect; this reduces the concentration of colistin needed and reduces the likelihood of side effects. Second, the spectrum of the second compound is expanded and resistance is unlikely since this second drug is normally used against a different set of bacteria. Furthermore, the synergistic effect of colistin and a second antibiotic has been shown to continue even after the emergence of colistin resistance. Colistin increases the permeability of the outer membrane by binding to lipid A, a vital component of lipopolysaccharide. Resistance to this drug is mediated by mutations in the *lpxA* gene, which prevent the synthesis of lipid A. The consequent impairment of lipopolysaccharide synthesis renders the cell hyperpermeable and thus more susceptible to other antibiotics [23]. While many examples of inhibitors to multidrug resistance pumps such as AcrAB-TolC or MexAB-OprM have been isolated from medicinal plants, compounds

with sufficient affinity to many types of clinically relevant efflux has not yet been found.

Aside from increasing the efficacy of an antibiotic by increasing its intracellular concentration, modifying cellular physiology with a second compound has been shown to achieve the same effect. This is because many antibiotics have been observed to be most effective on dividing or metabolically active cells. In Chapter 4, we add to the understanding of how physiology and antibiotics are linked by describing how the induction of bacterial stasis can reduce killing by these classes of antibiotics. Similarly, cells in a slow-growing persister state are protected from this bactericidal class of antibiotics. The reverse, that is increasing the effect of an antibiotic by increasing a cell's metabolic activity, has recently been described [24].

Unlike quinolone or beta lactam drugs that are incorporated as bacteria divide, the entry of aminoglycosides relies on proton-motive force that is generated during metabolism. In seeking to exploit this characteristic, Allison and colleagues performed a screen of carbon sources that could aid in the killing of dormant cells by increasing their metabolic activity. They found that compounds that enter upper glycolysis (glucose, mannitol, and fructose) potentiated the *in vitro* killing of persisters by aminoglycosides. Furthermore, they demonstrate the clinical relevance of this strategy by verifying the ability of gentamicin and mannitol to treat a biofilm-associated infection in a mouse model. While gentamicin alone had no effect, the combination of gentamicin and mannitol reduced the number of cells in the biofilm by 1.5 orders of magnitude. It is important to note, however, that there was no evidence that mannitol or the other effective metabolites lead to the stimulation of division. Their results demonstrate how a more complete understanding of the role metabolism plays in mediating killing by antibiotics could benefit the treatment of recurrent infections.

**Antibiotic combination therapy can overcome resistance**

Currently, there is only one example of an antibiotic pair that is able to kill cells bearing resistance mutations to both drugs. Like the other penicillin antibiotics, resistance to oxacillin can be mediated by the production of alternative penicillin-binding proteins (PBPs), which are involved in the synthesis of peptidoglycan, a major cell wall component. Specifically for oxacillin, the presence of this drug induces the expression of the *mecA* gene and the production of PBP2a. In a completely unrelated fashion, vancomycin also targets the cell wall by binding to the D-alanine-D-alanine peptide of peptidoglycan precursors and preventing their assembly into stable polymers. Resistance to this antibiotic is guided by a two-component regulatory system that controls the *vanHAX* operon, which in turn synthesizes alternative precursors ending in D-alanine-D-lactate. Interestingly, the unique mechanism of synergy reported for oxacillin

and vancomycin does not occur in sensitive or singly resistant bacteria, but only in strains containing both resistance mechanisms. Susceptibility to killing by these two drugs is the result of PBP2a being unable to accommodate peptidoglycan precursors ending in D-alanine-D-lactate. The simultaneous application of both drugs, therefore, induces both the production of PBP2a and the altered peptidoglycan precursor, which results in synergy [25].

Although this case will have few if any correlations beyond these particular antibiotics and mutations, this finding serves as a demonstration for how combination therapy can be used to overcome existing multidrug resistance. Over the course of this dissertation, I argue that examining the behavior of bacteria exposed to antibiotics could lead to strategies that aim for a more attainable but no less important goal. In order to delay the evolution and spread of resistance, attention should be reoriented to include non-hospital environments where pathogens both compete with and benefit from environmental bacteria and the vast amount of resistance determinants they harbor. More effort should be made to determine the effect of antibiotic concentration on eliciting responses from bacteria, influencing mechanisms of resistance as well as mechanisms of their maintenance and spread, and how these relationships impact the population dynamics of resistant and sensitive bacteria. Taking inspiration from the successful treatment of other diseases such as HIV, combinations of multiple drugs or drugs with adjuvants should be reviewed to increase the number of available treatment options for more bacterial infections, reduce our dependency on constant novel drug development, and prolong the useful life of the antibiotics we have.

**An alternative paradigm on the physiological state of antibiotic resistance:**

# Antibiotic resistance only confers resistance to killing by an antibiotic.

There is no contention that antibiotics at varying concentrations induce a number of effects in sensitive bacteria. These include changes in fitness (ranging from the inhibition of growth, to death), or a wide range of responses (such as the morphological changes already discussed in this chapter). However, to the best of my knowledge there are no published studies that expressly consider antibiotics on the fitness and range of responses of resistant bacteria. A common feature in the experimental chapters of this dissertation all indicate that this area deserves increased attention.

In this section, I build upon the relationship of the fitness of resistant bacteria and antibiotic concentration that is repeatedly alluded to throughout this dissertation. Specifically, I propose that the evolution of antibiotic resistance only confers resistance to the killing effect of an antibiotic. Bacteria that become resistant, therefore, are still susceptible to other effects – genetic, physiologic, morphologic, or behavioral – that can be induced by the antibiotic. I consider how different mechanisms of resistance may facilitate such antibiotic-induced responses in resistant bacteria, how varying concentrations may play a role, and even how the location of the resistance determinant (either on the chromosome or on a plasmid) may influence this effect. I focus this position on medically relevant resistance in human pathogens, and suggest clinical situations in which this phenomenon may play an important role.

### Mechanisms of resistance only promote survival in high drug concentrations

As discussed in greater detail in Chapter 1, the cellular mechanisms that confer resistance can be grouped into two broad categories. These are mutations of the antibiotic target, or dedicated genes that lower drug concentration (via efflux, decreased membrane permeability, or enzymatic inactivation).

For the first class of resistance mechanisms, cells that are resistant via mutations that affect the binding of an antibiotic to its cellular target may conceivably "not see" the drug at all. In Chapters 2 and 3, I provide evidence suggesting that this may not always be the case. In both chapters, I utilize bacteria that are resistant to streptomycin via mutations on *rpsL*, a gene that encodes the S12 subunit of the bacterial ribosome. I show that the fitness of resistant bacteria varies according to drug concentration. Furthermore, I found that the presence of high streptomycin concentrations could lead to higher growth rates of a streptomycin resistant strain in a new antibiotic-containing environment. These results support the hypothesis that a strain that has become resistant via mutating the target of the antibiotic can still be affected by the presence of the drug.

In comparison to resistance mechanisms that lower intracellular concentration, this first group of mutations are found on essential genes and they may introduce pleiotropic effects on normal cell physiology (i.e., restrictive streptomycin resistance mutations increase the accuracy of protein elongation). Furthermore, it is also possible that an antibiotic may be able to bind and affect two or more different cellular targets. Binding to one of these targets may lead to death, while binding to the second target may result in the generation of a different response. The detection of this second response may be masked by the death of a sensitive cell. Strains that are resistant via a mutation on the death-inducing target, however, will not die in the presence of this drug but will still have a viable second target by which to produce a second response. Predicting the type and variety of responses induced by an antibiotic for this type of resistance determinants, therefore, is likely more difficult.

In contrast, resistance determinants of the second type may in fact facilitate the phenomenon I propose. If we consider two genotypes that only differ by the presence of an efflux pump, the resistant strain will survive in high drug concentrations by lowering the intracellular antibiotic concentration to below the killing threshold. This strain, however, has cellular machinery identical to that of the susceptible genotype and is therefore responsive to the presence of sub-inhibitory antibiotics.

**Determining the role of antibiotic concentration**

A recurring message across the results presented in this dissertation is the importance of antibiotic concentration in mediating a number of effects – such as producing the non-monotonic dose-response curves of streptomycin resistant *Salmonella*, or the ability of sub-inhibitory levels of bacteriostatic drugs to cause diverse changes in division and elongation rates.

The model I propose suggests that bacteria that have become resistant do not lose the ability to produce responses to sub-inhibitory levels of antibiotic. Testing this aspect of the model would be straightforward due to the many documented responses of bacteria to low concentrations of drug. As an example, the production of cytolethal distending toxin by *C. jejuni* has been found to be potentiated by sub-inhibitory concentrations of erythromycin or ciprofloxacin [26]. Measuring the toxigenic effect of a range of drug concentrations against sensitive and resistant *C. jejuni* could help support this model.

**Plasmid-mediated regulatory complexity**

In relation to antibiotic resistance, one of the most recognized features of plasmids is their ability to increase the diversity of a bacterial population by facilitating horizontal transfer and recombination across and between bacterial species [27–30]. A complementary role these extra-chromosomal elements play

is the increased adaptability and responsiveness they confer on their hosts by virtue of the variety of genes they carry, as well as the additional regulatory complexity they can impose [31,32]. This relationship between the myriad of plasmid functions and the signal transduction systems encoded by the plasmid to influence both the plasmid itself and the chromosome is a field of intense study in a number of pathogens.

*Bacillus anthracis,* the Gram-positive, spore-forming agent of anthrax, serves as an interesting example to illustrate this point. A virulent strain of *B. anthracis* utilizes two large virulence plasmids, pXO1 and pXO2, that have commingled with chromosomal regulatory systems to coordinate its pathogenic life history. Infection begins with the inhalation or ingestion of spores. Although spores facilitate survival in harsh environmental conditions, the cell must transition into a vegetative state to avoid the immune system [33,34]. The precise order of signaling events and responsible machinery has yet to be completely elucidated, but the present knowledge suggests a complicated regulatory network underlies the disease cascade. Chromosomally encoded systems that sense cues such as $CO_2$ – bicarbonate (a marker of the mammalian host environment) or systems that initiate the stress response have been co-opted to increase expression of the two essential plasmid-encoded virulence factors, the anthrax toxin and capsule [35–37]. Correspondingly, regulators of plasmid expression have been found to influence the transcription of chromosomal genes [38].

Antibiotic-induced responses may be catalyzed in a similar fashion. A plasmid-encoded, two-component regulatory system may respond to the presence of a drug to express resistance genes, as well as other genes located on either the plasmid or the chromosome.

**Peeling back the overlapping, interlocking layers of antibiotic resistance**

Antibiotic resistance can be achieved by a variety of genetic and non-inherited mechanisms. The many genetic changes associated with resistance may conceivably constitute only a fraction of the mechanisms responsible for bacterial survive in the presence of antibiotics.

Currently, there are no precise reports on the disease burden attributed to non-inherited forms of resistance. Due to the types of pathogens that are known to utilize these mechanisms however, it is clear that this number is very large. The difficulty in treating infections with *M. tuberculosis* is primarily due to the non-growing, dormant state that reduces the efficacy of antibiotic therapy [39]. Behaviors such as swarming, bacterial group motility over a surface, have also been implicated in resistance to multiple drugs by pathogens such as *Salmonella* [40]. *Pseudomonas aeruginosa* exemplifies a perfect storm of effects that all result in non-inherited resistance. In this important opportunistic pathogen, regulatory elements have been described that respond to antibiotics to transiently in-

crease survival during antibiotic exposure, induce swarming, and the establishment of a biofilm [41]. As a last example in this non-exhaustive list, the formation of persisters is implicated as the root of recurring infections such as those caused by *S. aureus* [42]. In contrast to resistance, this small subpopulation of cells that remain after antibiotic exposure can only resume growth in the absence of drug and the resulting population is as sensitive as the initial population [43].

It is important to recognize that these different, non-inherited mechanisms likely work in concert to promote survival and even lead to genetic resistance. Growth in a biofilm has been found to result in endogenous oxidative stress that induces double-strand breaks in DNA – genetic diversity is generated when these breaks are repaired by a mutagenic mechanism utilizing recombinatorial DNA repair genes [44]. Interestingly, persistence may also lead to increased diversity and the emergence of genetic resistance [45]. Traditionally thought of as a metabolically dormant state, this is now challenged by an increasing amount of evidence suggesting that persistence is initiated and actively maintained by intracellular stress responses [46]. The induction of this stress response network can lead to heritable resistance via mutagenesis or the horizontal dissemination of mobile genetic elements bearing resistance genes [15,47].

The paradigm I propose, that antibiotic resistance is only resistance to killing and that resistant bacteria may still respond to antibiotics in a variety of ways, may similarly act in collaboration with different mechanisms that promote survival during drug exposure. This hypothesis clearly requires rigorous experimentation to determine when, to what extent, and in which organisms this may exist. What is clear, however, is that many bacterial responses that contribute to the virulence and survival of pathogens are only revealed in stressful environments. The presence of antibiotics is one such important environment. The processes that emerge are closely linked to dynamic regulatory networks that coordinate adaptation and ultimately lead to virulence, persistence, and may even foster the evolution of resistance in sensitive members of the population. Understanding these responses, therefore, can aid in the identification of new drug targets and the development of more effective strategies to control the problem of antibiotic resistance.

# References


1. Smith DL, Dushoff J, Morris JG (2005) Agricultural antibiotics and human health. PLoS Med 2: e232. doi:10.1371/journal.pmed.0020232.
2. Canton R (2009) Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin Micrbiol Infect 15: 20–25.
3. Sengupta M, Austin S (2011) Prevalence and Significance of Plasmid Maintenance Functions in the Virulence Plasmids of Pathogenic Bacteria. Infect Immun 79: 2502–2509. doi:10.1128/IAI.00127-11.
4. Johnson AP, Woodford N (2013) Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. J Med Microbiol 62: 499–513. doi:10.1099/jmm.0.052555-0.
5. Colomer-Lluch M, Jofre J, Muniesa M (2011) Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PloS One 6: e17549. doi:10.1371/journal.pone.0017549.
6. Torres-Cortés G, Millán V, Ramírez-Saad HC, Nisa-Martínez R, Toro N, et al. (2011) Characterization of novel antibiotic resistance genes identified by functional metagenomics on soil samples. Environ Microbiol 13: 1101–1114. doi:10.1111/j.1462-2920.2010.02422.x.
7. Goodman A, Kallstrom G, Faith J, Reyes A, Moore A, et al. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc Natl Acad Sci USA 108: 6252–6257. doi:10.1073/pnas.1102938108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1102938108.
8. Hastings P, Rosenberg S, Slack A (2004) Antibiotic-induced lateral transfer of antibiotic resistance. Trends Microbiol 12: 401–404. doi:10.1016/j.tim.2004.07.001.
9. Rolain JM, Fancello L, Desnues C, Raoult D (2011) Bacteriophages as vehicles of the resistome in cystic fibrosis. J Antimicrob Chemother 66: 2444–2447. doi:10.1093/jac/dkr318.
10. Fancello L, Desnues C, Raoult D, Rolain JM (2011) Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fi-

brosis sputum microbiota. J Antimicrob Chemother 66: 2448–2454. doi:10.1093/jac/dkr315.

11. Sandegren L, Andersson DI (2009) Bacterial gene amplification: implications for the evolution of antibiotic resistance. Nat Rev Microbiol 7: 578–588. doi:10.1038/nrmicro2174.

12. Koskiniemi S, Pränting M, Gullberg E, Näsvall J, Andersson DI (2011) Activation of cryptic aminoglycoside resistance in Salmonella enterica. Mol Micobiol: 1–15. doi:10.1111/j.1365-2958.2011.07657.x.

13. Justice SS, Hunstad DA, Cegelski L, Hultgren SJ (2008) Morphological plasticity as a bacterial survival strategy. Nat Rev Microbiol 6: 162–168.

14. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, et al. (2004) SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science 305: 1629–1631. doi:10.1126/science.1101630.

15. Beaber JW, Hochhut B, Waldor MK (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427: 72–74. doi:10.1038/nature02241.

16. Chen K, Sun GW, Chua KL, Gan Y (2005) Modified Virulence of Antibiotic-Induced Burkholderia pseudomallei Filaments. Antimicrob Agents Chemother 49: 1002–1009. doi:10.1128/AAC.49.3.1002.

17. Pena-Miller R, Laehnemann D, Jansen G, Fuentes-Hernandez A, Rosenstiel P, et al. (2013) When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. PLoS Biol 11: e1001540. doi:10.1371/journal.pbio.1001540.

18. Jawetz E, Gunnison J (1953) Antibiotic synergism and antagonism: an assessment of the problem. Pharmacol Rev 5: 175–192.

19. Chait R, Craney A, Kishony R (2007) Antibiotic interactions that select against resistance. Nature 446: 668–671. doi:10.1038/nature05685.

20. Falagas ME, Grammatikos AP, Michalopoulos A (2008) Potential of old-generation antibiotics to address current need for new antibiotics. Expert Rev Anti Infect Ther 6: 593–600. doi:10.1586/14787210.6.5.593.

21. Aoki N, Tateda K, Kikuchi Y, Kimura S, Miyazaki C, et al. (2009) Efficacy of colistin combination therapy in a mouse model of pneumonia caused by multidrug-resistant Pseudomonas aeruginosa. J Antimicrob Chemother 63: 534–542. doi:10.1093/jac/dkn530.

22. Gordon NC, Png K, Wareham DW (2010) Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multidrug-resistant strains of Acinetobacter baumannii. Antimicrob Agents Chemother 54: 5316–5322. doi:10.1128/AAC.00922-10.

23. Moffatt JH, Harper M, Harrison P, Hale JDF, Vinogradov E, et al. (2010) Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother 54: 4971–4977. doi:10.1128/AAC.00834-10.

24. Allison K, Brynildsen M, Collins J (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473: 216–220. doi:10.1038/nature10069.

25. Périchon B, Courvalin P (2006) Synergism between beta-lactams and glycopeptides against VanA-type methicillin-resistant Staphylococcus aureus and heterologous expression of the vanA operon. Antimicrob Agents Chemother 50: 3622–3630. doi:10.1128/AAC.00410-06.

26. Ismaeel AY, Senok AC, Bindayna KM, Bakhiet M, Al Mahmeed A, et al. (2005) Effect of antibiotic sub inhibitory concentration on cytolethal distending toxin production by Campylobacter jejuni. J Infect 51: 144–149. doi:10.1016/j.jinf.2004.09.012.

27. Ammann A, Neve H, Geis A, Heller KJ (2008) Plasmid transfer via transduction from Streptococcus thermophilus to Lactococcus lactis. J Bacteriol 190: 3083–3087. doi:10.1128/JB.01448-07.

28. Wiedenbeck J, Cohan FM (2011) Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiol Rev 35: 957–976. doi:10.1111/j.1574-6976.2011.00292.x.

29. Matic I, Rayssiguier C, Radman M (1995) Interspecies gene exchange in bacteria: the role of SOS and mismatch repair systems in evolution of species. Cell 80: 507–515.

30. Walsh TR (2006) Combinatorial genetic evolution of multiresistance. Curr Opin Microbiol 9: 476–482. doi:10.1016/j.mib.2006.08.009.

31. Cantón R, Ruiz-Garbajosa P (2011) Co-resistance: an opportunity for the bacteria and resistance genes. Curr Opin Pharmacol 11: 477–485. doi:10.1016/j.coph.2011.07.007.

32. Perego M, Hoch JA (2008) Commingling regulatory systems following acquisition of virulence plasmids by Bacillus anthracis. Trends Microbiol 16: 215–221. doi:10.1016/j.tim.2008.01.010.

33. Kang TJ, Fenton MJ, Weiner MA, Hibbs S, Basu S, et al. (2005) Murine Macrophages Kill the Vegetative Form of Bacillus anthracis, Infect Immun 73: 7495–7501. doi:10.1128/IAI.73.11.7495.

34. Hu H, Sa Q, Koehler TM, Aronson AI, Zhou D (2006) Inactivation of Bacillus anthracis spores in murine primary macrophages. Cell Microbiol 8: 1634–1642. doi:10.1111/j.1462-5822.2006.00738.x.

35. Dai Z, Sirard JC, Mock M, Koehler TM (1995) The atxA gene product activates transcription of the anthrax toxin genes and is essential for virulence. Mol Microbiol 16: 1171–1181.

36. Koehler TM, Dai Z, Kaufman-Yarbray M (1994) Regulation of the Bacillus anthracis Protective Antigen Gene : CO2 and a trans-Acting Element Activate Transcription from One of Two Promoters. J Bacteriol 176: 586–595.

37. Chitlaru T, Gat O, Gozlan Y, Ariel N, Shafferman A (2006) Differential Proteomic Analysis of the Bacillus anthracis Secretome : Distinct Plasmid and Chromosome CO 2 -Dependent Cross Talk Mechanisms Modulate Extracellular Proteolytic Activities. J Bacteriol 188: 3551–3571. doi:10.1128/JB.188.10.3551.

38. Bourgogne A, Drysdale M, Hilsenbeck SG, Peterson SN, Koehler TM (2003) Global Effects of Virulence Gene Regulators in a Bacillus anthracis Strain with Both Virulence Plasmids. Infect Immun 71: 2736–2743. doi:10.1128/IAI.71.5.2736.

39. Baek S, Li AH, Sassetti CM (2011) Metabolic Regulation of Mycobacterial Growth and Antibiotic Sensitivity. PLoS Biol 9: e1001065. doi:10.1371/journal.pbio.1001065.

40. Butler MT, Wang Q, Harshey RM (2010) Cell density and mobility protect swarming bacteria against antibiotics. Proc Natl Acad Sci USA 107. doi:10.1073/pnas.0910934107.

41. Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock REW (2008) Induction by cationic antimicrobial peptides and involvement in intrinsic polymyxin and antimicrobial peptide resistance, biofilm formation, and swarming motility of PsrA in Pseudomonas aeruginosa. J Bacteriol 190: 5624–5634. doi:10.1128/JB.00594-08.

42. Chong Y, Park S, Kim H, Kim E, Kim M, et al. (2013) Persistent Staphylococcus aureus bacteremia: a prospective analysis of risk factors, outcomes, and microbiologic and genotypic characteristics of isolates. Medicine (Baltimore) 92: 98–108.

43. Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol Rev 33: 704–717. doi:10.1111/j.1574-6976.2008.00156.x.

44. Boles BR, Singh PK (2008) Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proc Natl Acad Sci USA 105(34): 12503-8.

45. Cohen NR, Lobritz M a, Collins JJ (2013) Microbial persistence and the road to drug resistance. Cell Host Microb 13: 632–642. doi:10.1016/j.chom.2013.05.009.

46. Poole K (2012) Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol 20: 227–234. doi:10.1016/j.tim.2012.02.004.

47. Walsh C (2000) Molecular mechanisms that confer antibacterial drug resistance. Nature 406: 775–781. doi:10.1038/35021219.

# Paolo Santiago S. OCAMPO


| | |
|---|---|
| phone / email | +41 (0)76 680 3028 / paolo.ocampo@env.ethz.ch |
| date of birth | July 15, 1982 |
| country of citizenship | Philippines |


## Work experience and Education

### PhD candidate in Biology 2009 - 2013

- University of Zürich, Zürich, Switzerland
- Research topics: evolution of antibiotic resistance, single cell time-lapse microscopy, antibiotic interactions, microfluidics
- Advisors: Sebastian Bonhoeffer, Martin Ackermann, Leo Eberl

### Medical student 2006 – present

- Emory University School of Medicine, Atlanta, GA, USA

### Research Technician 2005 - 2006

- Laboratory of Bruce Levin, Dept. of Biology, Emory University, Atlanta, GA
- Studied the effect of biofilms on antibiotic treatment and the emergence of antibiotic resistance
- Investigated the role of phage in the modulation of *V. cholerae* epidemics

### Bachelor of Science (summa cum laude) 2002 - 2005

- Emory University, Atlanta, GA, USA
- Double major in Anthropology and Human Biology, and Biology
- Honors thesis supervised by George Armelagos, Dept. of Anthropology, and performed at the Centers for Disease Control and Prevention (CDC), Atlanta, GA.
- Characterized the *arp* gene of the different pathogenic *Treponema* to help determine the evolutionary trajectory of modern venereal syphilis

### Research Technician 2004 – 2005

- Steiner Laboratory, Division of AIDS, STD, and TB Laboratory Research, Centers for Disease Control and Prevention, Atlanta, GA
- Analyzed the *arp* gene sequence from pathogenic *Treponema pallidum* strains
- Assisted in laboratory maintenance

### Research Technician 2002 - 2003

- Bhat Laboratory, Department of Cell Biology, Emory University School of Medicine, Atlanta, GA
- Analyzed *Drosophila* neuronal guidance protein interactions
- Performed a deficiency screen for regulators of neuronal guidance and transport

### Undergraduate University studies 2000 - 2002

- University of the Philippines, Manila
- Undergraduate studies in Biochemistry

### High School Diploma graduated 2000

- Xavier School, San Juan, Metro Manila, Philippines

# Scientific publications

• The fitness landscapes of antibiotic resistance mutation and its compensatory mutation partners

**Ocampo PS**, Engelstaedter J, Schulz zur Wiesch P, Ackermann MA, Bonhoeffer S. In preparation for PLoS Pathogens

• Antagonism is prevalent between bacteriostatic and bactericidal antibiotics

**Ocampo PS**, Lazar V, Bogos B, Abel zur Wiesch, P, Pal C, Ackermann MA, Bonhoeffer S. In preparation for PLoS Pathogens

• An experimental study of the population and evolutionary dynamics of Vibrio cholerae O1 and the bacteriophage JSF4

Wei Y, **Ocampo PS,** Levin BR. Proc Biol Sci (2010) 277(1698):3247-54

• On the Origin of the Treponematoses: A Phylogenetic Approach

Harper KN, **Ocampo PS,** Steiner BM, George RW, Silverman MS, Bolotin S, Pillay A, Saunders NJ, Armelagos GJ. PLoS Negl Trop Dis (2008) 2(1):e148

• The sequence of the acidic repeat protein (*arp*) gene differentiates venereal from nonvenereal *Treponema pallidum* subspecies, and the gene has evolved under strong positive selection in the subspecies that causes syphilis

Harper, KN, Liu, H, **Ocampo, PS,** Steiner, BM, Martin, A, Levert K, Wang, D, Sutton, M, Armelagos GJ. FEMS Immunol Med Microbiol (2008) 53(3):322-32

• The origin of syphilis: a phylogenetic approach suggesting New World origin.

Harper, KN, **Ocampo, PS,** Steiner, BM, George, RW, Silverman, MS, Bolotin, S, Pillay, A, Armelagos GJ. Am J Phys Anthro (2006) :101

• Towards a solution to the syphilis enigma: trend in the arp gene suggests evolutionary relationships of the treponemes

**Ocampo, PS,** Liu, H, Harper, KN, George, RW, Armelagos, GJ, Steiner, BM. Am J Phys Anthropol (2006) :140

• The myth of syphilis: The natural history of treponematosis in North America

Armelagos GJ, Harper, KN, **Ocampo, PS.** Evol Anthro (2005) 14:240-242

# Skills

Fluent in English and Filipino. Conversational Mandarin. Basic German and French.

General molecular biology and microbiology protocols. Reproducible growth and time-lapse microscopy of living cells. Microfluidics. Genetic modification of *E. coli* and *S. enterica*.

Extraction and concentration of aDNA. Osteological analysis of skeletal material.